A palm oil substitute and care product emulsions from a yeast cultivated on waste resources

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A PALM OIL SUBSTITUTE AND CARE PRODUCT EMULSIONS FROM A YEAST CULTIVATED ON WASTE RESOURCES

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CONTENTS

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Finally, thanks again to Chris, Jordan and my mum.

DECLARATION

This dissertation is the result of my own work and includes nothing, which is the outcome of work done in collaboration except where specifically indicated in the text. It has not been previously submitted, in part or whole, to any university or institution for any degree, diploma, or other qualification.

Collaborations were as follows:

Chapter 2

Fabio Santomauro (University of Bath) prepared and harvested cultivations for figures 2.1, 2.2, 2.4, 2.5, 2.6, 2.7 and 2.16, I did the lipid extraction, lipid analysis, cell dry mass measurements and sugar analysis and assisted with sampling. Joe Ronayne developed the dilute acid hydrolysis method and provided data for the water-hydrolysed sample in Table 2.5 and the straw composition.

Chapter 3

Lilia Zenker (University of Hamburg) developed the hydrolysis method and provided three samples of straw hydrolysates. I prepared and harvested the cultivations and collected all the data.

Christopher Ibenegbu (University of Bath) provide furfural contents.

Chapter 4

Alice Fan and colleagues at the University of York prepared the microwaved straw hydrolysates, provided HPLC data for oligosaccharides, rhamnose, levoglucosan, fructose, mannose, galactose. These collaborators also provided CHN and ICP-MS data.

For Figure 14 Fabio Santomauro prepared and harvested the cultivations and extracted the lipid. I prepared the hydrolysates.

Chapter 5

No collaborations.

ABSTRACT

Currently, the production of palm oil leads to the destruction of rainforest. A more sustainable source of lipids could be obtained using abundant lignocellulosic waste (e.g. wheat straw) as a source of carbon in the form of polysaccharides. Some species of oleaginous yeast, grown on sugars, can be made to accumulate between 20-80% of their biomass as oil and so offer a promising alternative to terrestrial crops.

In this thesis, the yeast *Metschnikowia pulcherrima* was selected for its resilience to contamination. Although not previously classified as oleaginous, a combination of low temperature and restricted nutrient availability prevented sporulation and consequently triggered levels of oil production in *M. pulcherrima* cultures of up to 47%. The potential of this yeast to produce lipids inexpensively on waste resources was investigated.

This yeast was grown under non-sterile conditions at pilot scale with minimal temperature control. The possibility of growing *M. pulcherrima* on lignocellulose was studied on models and showed that it was tolerant to a range of sugars and inhibitors commonly found in hydrolysed lignocellulose. The yeast produced 6.04 g L^{-1} lipid when cultivated on a mixture of hexoses and pentoses.

This was corroborated by demonstrating that the yeast could be cultured on oligomers and sugars produced by hydrolysing wheat straw. Evidence of cellulase production was observed, and this was utilised in a process involving mildly pretreated wheat straw, using a range of pretreatment processes and culture conditions to yield a maximum of 1.12 g L^{-1} lipid. The usefulness of the products of this cultivation in forming oil in water emulsions was evaluated, and some evidence of surface-active effects was found. Overall, *M. pulcherrima* was found to have phenotypes that would be highly beneficial in reducing the capital and running costs of a putative lipid production process.

LIST OF ABBREVIATIONS, SYMBOLS AND ACRONYMS

ACL – ATP citrate lyase

AHWS – Acid hydrolysed wheat straw

AMP – Adenosine monophosphate

ATCC - American Type Culture Collection, USA.

ATHUM – ATHUM Culture Collection of Fungi, Greece

ATP – Adenosine triphosphate

CBS - Centraalbureau voor Schimmelcultures, (culture collection) Utrecht, Netherlands.

CBP – Consolidated bioprocessing

C. curvatus – Cryptococcus curvatus.

CH – (Culture collection) Laboratory of Energy and Biochemical Engineering, Guangzhou Institute of Energy Conversion, Chinese Academy of Sciences

CHN – Carbon Hydrogen Nitrogen analysis

CI – Creaming Index

CI_{rel} – Relative Creaming Index

EDTA - Ethylene Diamine Tetraacetic Acid

FAME – Fatty acid methyl ester

4-HBA - 4-HydroxyHenzoic Acid

5-HMF - 5-(HydroxyMethyl)Furfural

HPLC – High Performance Liquid Chromatography

ICP-MS - Inductively Coupled Plasma Mass Spectrometry

M. pulcherrima - *Metschnikowia pulcherrima*

LC –Lipid Content

LHW – Liquid Hot Water

LY – Lipid Yield

ME – Malic enzyme

NAD+ - Nicotinamide adenine dinucleotide

NADPH- Reduced Nicotinamide Adenine Dinucleotide Phosphate

NADP+ - Nicotinamide Adenine Dinucleotide Phosphate

NCYC - National Collection of Yeasts Cultures, Norwich, UK.

NMR – Nuclear Magnetic Resonance spectroscopy

NREL – National Renewable Energy Laboratory

NRRL – Northern Regional Research Lab, (now United States Department of Agriculture-Agricultural Research Culture Collection, Peoria, Iowa, USA;

OD – Optical Density

OMP – Original *Metschnikowia Pulcherrima*

2PE – 2-phenylethanol

RID – Refractive Index Detector

RSPO – Roundtable on Sustainable Palm Oil

rpm – revolutions per minute

S. cerevisiae – *Saccharomyces cerevisiae*

SHF – Separate Hydrolysis and Fermentation

SFF – Simultaneous Saccharification and Fermentation

T – Temperature

t - Time

UCD-FST-University of California Davis, Food Science Technology, Phaff yeasts culture collection

UV – Ultraviolet (light)

WHWS – Water Hydrolysed Wheat Straw

X – Cell dry mass

YE- Yeast Extract concentration

YMM- Yeast Minimal Medium

YPD - Yeast extract, Peptone, Dextrose (glucose) yeast medium

YMS - Yeast extract, Mannitol, Sorbose (yeast medium)

1 Introduction

1.1 The need for a palm oil substitute

1.1.1 Palm oil

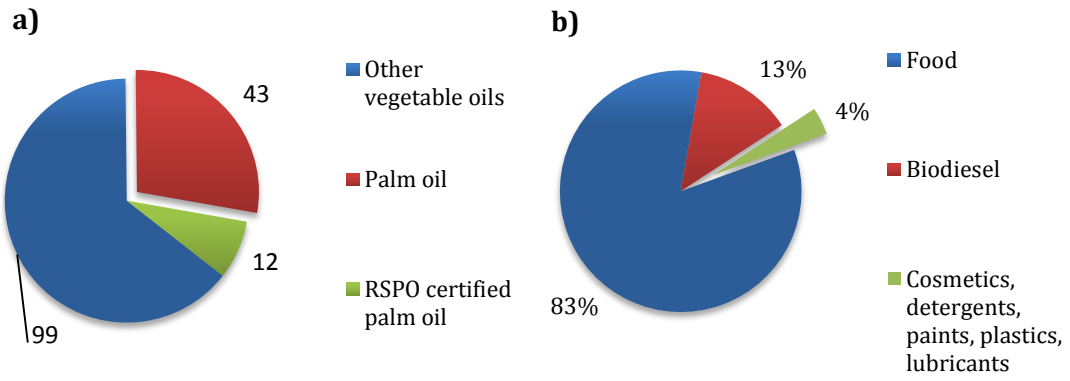


Figure 1.1. a) Vegetable oils produced (mt y^{-1}) b) Usage of vegetable oils.[1, 2]

Vegetable oils are a significant source of renewable, bio-based chemicals. In 2014, 162 m tonnes of vegetable oil were produced. The majority of the global vegetable oil stock is used as a foodstuff, 4 % is used as a platform chemical feedstock, and 13% used for biodiesel production (Figure 1.1b).[1]

Of these oils, the one of the greatest sources of vegetable oil is the oil palm tree. The palm oil this crop produces is particularly prized in food and in the care product industry for its thick texture and multitude of products that can be derived from it (Figure 1.1).[2] While the price fluctuates, generally palm oil retails for between US$ 700-1200 t^{-1}, which is lower than both rapeseed and soybean oil. This is partly because oil palms have the highest yield of oil per hectare of any oilseed (18.5 t ha^{-1}).[1, 3] The majority of production is in Indonesia and Malaysia,[1] and the minority being in Colombia, Ecuador, Nigeria, Cote d'Ivoire, and

Papua New Guinea, among other nations.[4] Production of the oil has doubled in the last decade and currently stands at 55 million tonnes per year and is projected to continue increasing at around 4% per year. This is due to the increased demand for biofuels and food oils.[1] In 2007 Malaysia and Indonesia announced that they would reserve 40 percent of their palm oil output for biodiesel.[4] It has provided a huge boost to their economies and it is estimated that 4.5 million people in Indonesia and Malaysia alone are directly employed by the industry.[2, 4]

Although palm oil is renewable, the sustainability of increasing production is questionable, as the oil palm crop can only grow in the same climate as the tropical rainforests. As such the increase in oil palm tree plantations has led to widespread destruction of these rainforests. For example, on the Indonesian island of Sumatra approximately 7.5 MHa of rainforest (70% of the total) was cut down between 1990 and 2010.[4-6] It is unclear exactly how much of this deforestation was explicitly incentivised by palm oil and not for timber or other agriculture. However, this reason is cited by those given government permits to clear forests, and around a third of the cleared land has now been converted to oil palm plantations.[4, 7, 8] Indonesia, for example, has increased the area used for oil palm plantations from 0.1 million hectares in 1960 to six million hectares in 2006, with full governmental support.[4] This loss of habitat for plants,[9] animals[7] and indigenous people[4] is compounded by an increase in carbon emissions (as well as air pollution) as rainforest is burned.[8, 10]

One study estimated that due to the release of carbon stored in peat in the rainforest that palm oil biodiesel would need between 83 and 423 years to repay this carbon debt (for peatland and non-peatland rainforest, respectively).[11] These carbon emissions due to land use change from deforestation are significant, and could total up to 15% of all global carbon emissions worldwide.[12, 13]

Presently rainforest clearance is continuing despite efforts from governments and groups such as the Roundtable on Sustainable Palm Oil (RSPO) who attempt to solve these issues by encouraging producers of palm oil to grow their crops more sustainably and certifying the farmers when they meet their standards.[5, 14] The organisation also persuades buyers of palm oil to demand that it is RSPO certified. After working for over ten years and securing the support of most of the largest purchasers of palm oil, only 13 million tonnes (or 3.44 million hectares) out of approximately 57 million tonnes were certified at the time of writing (Figure 1.1a).[15]

Oil palm trees are farmed as a monoculture of one species, *Elais guineensis*, native to West Africa. The oil is separately extracted from both the mesocarp (flesh) and the kernel (seed) of oil palm fruits (Figure 1.2). Both components are then fractionated into a liquid

fraction (palm olein) and a solid fraction (palm stearin) and may be chemically derivatised depending on the application. Palm oil is a highly saturated vegetable oil consisting of mostly palmitic and oleic acid (Table 1.1).

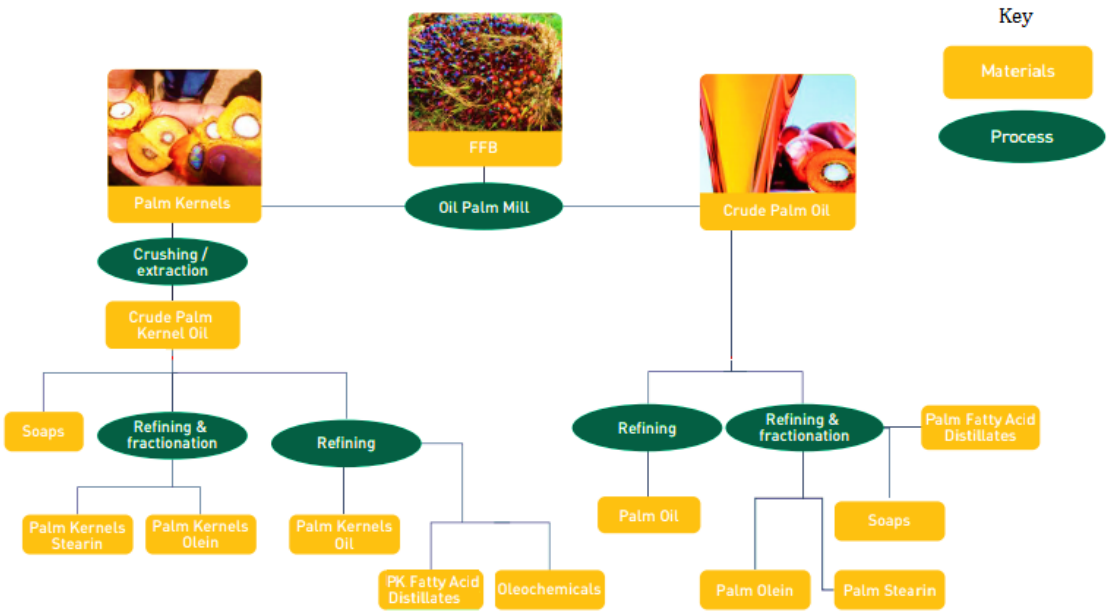


Figure 1.2. Processing of oil palm fresh fruit bunches (FFB) into crude palm oil, crude palm kernel oil, and their fractionation into stearin and olein fractions, soaps, fatty acid distillates and oleochemical derivatives. Adapted from Unilever.[16]

Lipid number	**C10:0**	**C12:0**	**C14:0**	**C16:0**	**C18:0**	**C18:1**	**C18:2**	**other**
Fatty acid name	Capric acid	Lauric acid	Myristic acid	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	
Palm stearin	5.00 ±0.13	2.79 ±0.16	6.63 ± 0.25	42.24 ± 0.34	4.46 ± 0.09	30.39 ± 0.52	5.99 ± 0.31	2.50 ± 0.08
Palm olein	5.27 ±0.18	2.73 ±0.11	5.33 ±0.19	31.18 ±0.26	3.89 ±0.14	39.42 ±0.27	9.76 ±0.44	2.42 ±0.11

Table 1.1. Composition by % of fatty acids in the two main fractions of palm oil – palm stearin and palm olein from a single random sample.[17]

Although tropical forests cannot be easily replaced, palm oil is not irreplaceable. Finding a replacement for palm oil would allow the people who are already generating an income from palm oil production to continue doing so, yet future growth in production could instead come from a new source that does not have the same negative environmental and social impacts.

1.1.2 Uses of palm oil in care product emulsions

Mineral oil has traditionally been used to supply the vast majority of the platform chemicals and fuels of the world.[9, 14-17] The first non-food use of palm oil was patented in 1897[18] and palm oil and its derivatives are now ubiquitous in the personal care product industry. Personal care products include moisturisers, shampoos, conditioners, hair products, skin products, face creams, and lotions. Palm oil products are multi-functional, serving as an emollient (to smooth and soften skin), skin or hair conditioner, surfactant (colloquially referred to as an emulsifier) and stabilising agent (Table 1.2, below).[19] As palm oil, like other vegetable oils, is principally composed of triglycerides, these can be hydrolysed into fatty acids, and may be hydrogenated to alcohols. These building blocks can then be made into a range of esters, amides or complexed with metals. Palm oil is valued in this industry for being "renewable" as it allows the partial or total replacement of mineral oil. It is also prized as a source of β-carotene, vitamin E and for being a non-irritant.[20] It has a melting point of around 33 °C, depending on the fatty acid profile, which contributes to its thick texture.

Personal care products are often composed of oil in water emulsions. Emulsions are liquid-liquid colloids, meaning that liquid droplets (ca. <1000 nm) of one phase (oil) are suspended in another phase (water) and do not settle due to gravity. Emulsions can be formed by shaking, stirring, or sonicating a mixture of aqueous and non-aqueous components at various speeds. Industrially, a homogeniser is often used. Emulsions are thermodynamically unstable compared to biphasic oil on water systems, and so oil droplets will coalesce unless kinetically stabilised. One method of kinetic stabilisation is using surfactants. A typical surfactant has a polar (hydrophilic) head and a non-polar (hydrophobic) tail, the tail being soluble in the oil phase, and the head being soluble in the water phase. This lowers the surface tension between the two layers. The surface tension is the free energy required to create new surface area (Jm^{-2}). Lowering the surface tension increases the number and decreases the size of the oil droplets that can form.[21,22]

Class of care product ingredient	Examples in class (common names)	Function of care product ingredient
Triglycerides	Palm kernel/oil/wax	Emollient, skin conditioner
Partially hydrolysed triglycerides	Glyceryl mono/di-oleate/palmitate	Emollient, emulsifier
Fatty acids	Palmitic acid, oleic acid	Emollient, emulsifier
Hydrogenated fatty acids	Hydrogenated palm acid	Opacifying agent
Fatty alcohols	Palm/palm kernel alcohol, cetaryl alcohol	Skin conditioning, emollient
Soaps –salts of fatty acids or complexes of fatty acids with metal ions	Sodium/potassium palm kernelate/palmate	Surfactant, emulsifier, cleansing agent
	Magnesium palmitate	Opacifying, viscosity controlling, anticaking agent
	Zinc dipalmitate	Deodorant
Esters of fatty acids and fatty alcohols	Sucrose palmitate, retinyl palmitate, ethyl/ethylhexyl palmitate, cetyl ester	Emulsifier, surfactant, skin conditioner, thickener.
	Ascorbyl mono/dipalmitate	Antioxidant
	Isopropyl palmitate	Antistatic, binding, emollient, solvent, skin conditioning
Fatty acid esters with polyethylene glycol	PEG-20 Oleate/palmitate	Emulsifying/surfactant
	Polypropylene glycol –15 – palmeth 60 – alkyl dicarbamate	Viscosity controlling
Quaternary ammonium ions fatty acid/alcohol groups	Ammonium palm kernel sulphates	Surfactant, cleansing, foaming
	Pyridoxine dipalmitate	Cationic surfactant, antistatic, skin, hair conditioner
Amides from fatty acids	Palm kernel amide	Emulsifying, surfactant, viscosity controlling
	Palmamidopropyl betaine	Antistatic, surfactant, skin/hair conditioning, cleansing,

Table 1.2. Care product ingredients derived from crude palm oil and palm kernel oil and their functions.[19]

As well as lowering the surface energy, surfactants stabilise oil in water emulsions by increasing the steric interactions between oil droplets. The size of the droplets affects macroscopic characteristics of the emulsion, such as viscosity, transparency and other rheological properties such as pouring behaviour and response to shear stress. Molecules that act as surfactants include fatty acids and the salts, esters, amides and alcohols formed from them (Table 1.2). Proteins and polypeptides can sterically stabilise colloids, such as those which stabilise the foam in beer and soft drinks.[21, 22]

Besides palm oil, other sources of renewable surfactants are sophorolipids, which are glycolipids synthesised by yeasts. [23, 24-26] These have the benefit of being biodegradable and have been successfully commercialised.[27] However, these do not bypass the need for triacylglycerides, as sophorolipids are formed from triacylglycerides added to the growth medium, so are more accurately described as biologically derivatised surfactants. Other by-products may also be derived from yeasts such as carotenoids, which are antioxidants.[28, 29]

1.1.3 Alternative feedstocks for a palm oil substitute

Biologically derived organic chemicals can come from a range of sources, typically characterised by the extent to which they compete with food crops. First generation sources are produced from edible crops that are grown to provide chemicals, rather than food. Examples of first generation oils are vegetable oils or microbial oils cultured on sugars derived from sugarcane or corn starch. Bioethanol from first generation sources is a commercial reality most notably in the USA and Brazil.[30] Despite being used heavily for biodiesel production in Europe, oil-producing crops such as soy and rape compete with agricultural land and so increase food prices and are widely accepted to be unsustainable in a world with a growing population.[11, 31-33]

Commonly the term 'second generation' is applied to feedstocks which are inedible, and do not have to be cultivated on agricultural land. They include lignocellulosic sources such as switchgrass agricultural waste, forestry waste and sustainably produced wood (see section 1.1.4).[34, 35] Second generation oils were thought to include the tree *Jatropha curcas* that yields oil filled seeds that could grow on marginal land. However, after trialling it in Africa and India, it transpired that in order to get enough oil for the crop to be worthwhile, it must be grown on fertile, agricultural land requiring fertiliser and water. This has limited the use of *J. curcas* to smaller scale hedgerows.[36, 37]

The term "third generation" is applied to non-terrestrial organisms that use the feedstock carbon dioxide, principally oil accumulating microalgae. Oils from microalgae have been

extensively studied.[38-48] The theoretical benefits of microalgae are significant – they accumulate lipid more rapidly (weeks vs. months) and at higher densities (around 20-50% by weight) than terrestrial oil crops (Table 1.3). The CO2 feedstock that they use is either inexpensive (from power plants) or free (atmospheric). They can be combined with wastewater treatment or grown in seawater to provide added sustainability benefits. However, despite decades of development several key disadvantages still limit the applicability of algae for lipid production. Microalgal cultures require nutrients, commonly fertilisers, that are problematic in terms of both cost and carbon footprint.[44] They are highly prone to being outcompeted by invasive species, meaning that cheaper, outdoor ponds open to the air are unviable.[49-51] Microalgae require light of suitable intensity and wavelength. Consequentially, if sunlight is to be used productivity is limited by the amount of light a potential site receives, limiting the locations where microalgae can be grown, and a large amount of land is required. Artificial lighting may be used in a photobioreactor, but this, as well as the need for temperature control (microalgae must be cooled during the day and heated at night) adds to the energy-related costs.[44, 50] The presence of a cell wall also makes extracting the oil energy intensive that can require a greater energy input than is stored within the oil produced.[40, 43, 45, 52] Overall, microalgae appear to be too expensive and too energy and carbon inefficient for commercial biodiesel production,[40, 44, 50] as evidenced by numerous oil companies, such as Shell and Exxon Mobil, halting their microalgal programmes.[53]

Crop	Oil yield (L ha^{-1})
Corn	172
Soybean	446
Canola	1190
Jatropha	1892
Coconut	2689
Oil palm	5950
Microalgae[a]	136,900
Microalgae[b]	58,700

Table 1.3. Yield of oil from some oil crops and microalgae with a) 70% lipid by weight b) 30% lipid by weight [38]

1.1.4 Lignocellulose as a feedstock for a palm oil substitute

Seemingly a more reasonable route to a palm oil replacement in personal care products is to use second-generation lignocellulosic biomass. Second generation lignocellulosic biomass such as agricultural stover, forestry waste, or dedicated energy crops (e.g. switchgrass) or bamboo (Miscanthus) demonstrate a lower carbon debt for land use change compared to palm oil, or other first generation sources, as well as eliminating the food vs. fuel issue.[54-56] Currently agricultural waste may be burnt, ploughed back into the soil, used as animal feed or removed from the land and landfilled. Due to short crop cycles rice straw is often burnt in the fields, resulting in airborne emissions hazardous to human health.[57] Burning is particularly harmful to the environment as it releases CO, particulate matter and NO_x, alongside CO_2.[57, 58] Not all agricultural residue is removable, as some must be allowed to be returned to the soil in order to limit nutrient loss and prevent soil erosion, and some is used as animal bedding[55, 59]

	Mass (Mt)	
Agricultural waste	**Europe**	**Global**
Rice straw	3.90	731.30
Wheat straw	132.59	354.34
Corn straw	28.61	203.61
Sugarcane bagasse	0.01	180.73
Total	165.11	1469.98

Table 1.4. Amounts of agricultural waste available in Europe and globally.[56]

Lignocellulose is far more abundant than first generation sugars. It has been estimated, for example, that in the US alone an additional 221 million tonnes agricultural residue could be sustainably harvested totalling one billion tonnes of first and second generation lignocellulose by 2030.[60] Agricultural waste is one of the largest and most sustainable sources of lignocellulose.[54-56] In Europe, there are an estimated 165 million tonnes lignocellulose available (Table 1.4).[61] One of the waste materials produced in the largest volumes in the UK is wheat straw, with up to 10 million tonnes produced annually.[59]

	Value (US $ t^{-1} biomass)
Palm oil†	700-1200
Average bulk chemical	1000
Transportation fuel	200–400
Cattle feed	70–200
Generating electricity	60–150
	Cost ($t^{-1} biomass)
Landfill	–400

Table 1.5. Value of potential lignocellulose biomass products, and costs of disposal per metric tonne. Adapted from Tuck et al[62] except for †palm oil values from World Bank between 2010- 2014[63].

Crucially, lignocellulose is estimated to be a cheaper source of sugars (US $0-80 t^{-1}) than first generation sources such as corn (US $100-300 t^{-1})[1] or sugar beet (US $200-600 t^{-1}),[63] particularly lignocellulose from agricultural or municipal wastes.[58,62] This means there is a potential for economic sustainability, provided the costs for converting lignocellulose to a palm oil substitute are low enough (Table 1.5.).

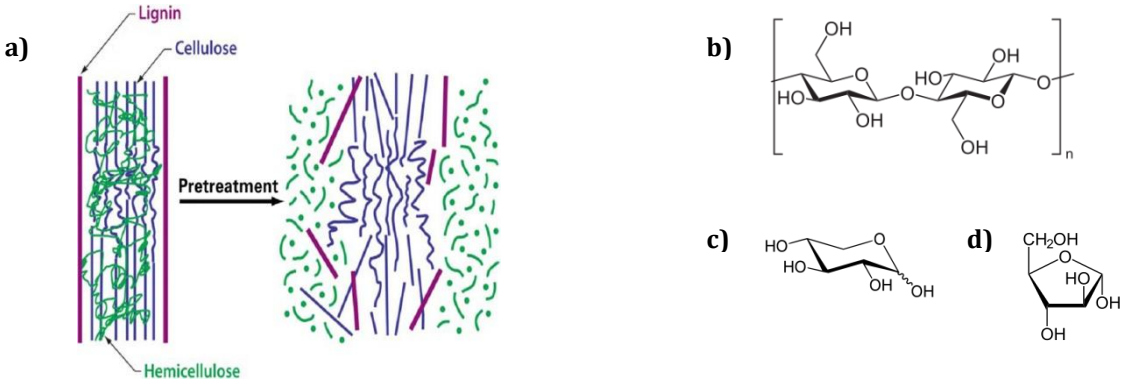


Figure 1.3. a) Schematic of the structure of lignocellulose and the effect of pre-treatment on this structure. b) Cellulose showing glucose monomers with 1-4-β-glycosidic link, c) xylose and d) arabinose - pentose sugars that are monomers of the polysaccharide hemicellulose.[34]

Dried plant biomass generally consists of at least 90% lignocellulose. The general structure of lignocellulose contains fibres made of cellulose, a polysaccharide consisting of glucose monomers linked by β-1,4-glycosidic bonds, which are bound by hydrogen bonds into a highly crystalline supramolecular structure. Hemicellulose fibres, a polysaccharide

primarily made from xylose and arabinose, are loosely bound around the cellulose fibres. The whole structure is enveloped by lignin – a polyphenolic compound (Figure 1.3).[34]

If the polysaccharides can be hydrolysed, the resulting hydrolysates of any plant biomass contain significant quantities of monomeric sugars. These monomers are principally glucose and xylose as well as minor proportions of arabinose, galactose, rhamnose, mannose, and in occasionally more exotic sugars – for example seaweeds contain sulfated sugars.[64] The exact composition of the hydrolysate depends on the lignocellulose sample, and the method of hydrolysis used (Table 1.6). These sugars can then be upgraded into oils, or other products of interest.

Category	Feedstock	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Agricultural waste	Wheat straw	35–45	20–30	8–15
	Corn cobs	45	35	15
	Rice straw	32–47	19–27	5–24
	Corn straw	42.6	21.3	8.2
	Sugarcane bagasse	65 (total carbohydrate)	18.4	3
Forestry waste	Hardwood stems	40-55	24-40	18-25
	Softwood stems	40-50	25-35	25-35
	Leaves	15-20	80-85	0
Energy crops	Coastal Bermuda grass	25	35.7	6.4
	Switchgrass	45	31.4	12
	Grasses	25-40	35-50	30-40
Municipal waste	Paper	85-99	0	0-15
	Sorted refuse	60	20	20

Table 1.6. Percentage cellulose, hemicellulose and lignin in lignocellulose. Remainder is ash and protein.[34, 56]

Currently, two main routes have been investigated to upgrade lignocellulose into oil – thermally or biologically. Thermal conversion by high temperature liquefaction usually gives a large range of products including bio-oils and biochar and is energy intensive, whereas biological conversion is less energy intensive and has the potential to extract valuable co-products. Both methods have their merits and it has been argued by Clark *et al.* that they should be combined in one biorefinery.[65]

1.2 Biotechnological applications of oleaginous yeasts

When selecting an organism with which to produce oil, yeasts have several advantages. Currently yeasts are used industrially to manufacture a wide range of chemicals for applications in food,[66] pharmaceuticals[67] and fuels.[68, 69] Compared to arable crops, production using yeasts is near continuous rather than annual (assuming that the entire lignocellulosic feedstock is not converted within a few months, or that multiple feedstocks are used) and is less labour intensive. As they have a short growth cycle[69] - yeasts have a duplication time of around two hours[70] so can reach maximum cell density in 12 -72 hours[23] - lipid productivity is theoretically greater (provided lipid yields are sufficient). This also gives them an advantage over moulds, which are slower growing, as well as being less tolerant of high osmotic pressure, metals and bacterial contamination. In addition the hyphae of moulds can lead to unacceptably high viscosities when grown in a fermenter.[71]

Compared to microalgae, (section 1.1.3 above), oleaginous yeasts have several benefits. Yeasts do not require light, reach a higher cell density in a shorter time (10-100 g L^{-1} in 3-7 days for yeasts[69] vs. 1-3 g L^{-1} in 1-7 weeks for microalgae[72]) and can produce a range of fermentation products as well as oils, all of which provide economic advantages for a potential industry. Accordingly, oil from yeasts (US $1200-3000)[50, 73] has been estimated to be far cheaper to produce than oil from microalgae (US $5600-21,000 t^{-1}).[50]

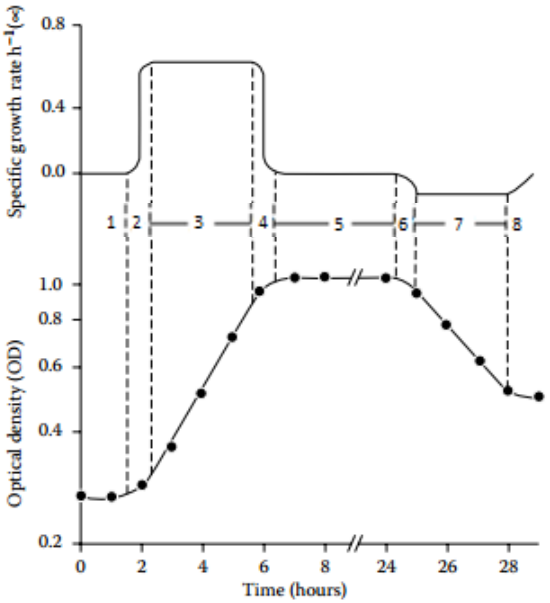
Generally, compared to bacteria, yeasts have a higher inhibitor tolerance,[74] and although bacteria are also capable of making sophorolipids(see 1.1.2 above), only a few strains of bacteria are known to make triglycerides, which are useful for emollients in care products or conversion to biodiesel.

Although there are approximately 1300 known species of yeasts,[75] this is estimated to be as little as 1% of the known extant species.[76] Although the genomes of the small number of yeasts that have been sequenced indicate that they possess around 6000 genes, there is great diversity in the physiology and biological niches inhabited by yeasts.[77] Yeasts are adapted to live in almost every habitat on the planet including the surface of fruit and the bottom of the sea.[77]

Oleaginous yeasts are defined as those that accumulate lipids at over 20%-70% of their cell dry mass in intracellular lipid globules.[23, 78-82] This value is called the lipid content (LC, %), the mass of the dried cells per unit volume of growth medium is defined as the cell dry mass (X, g L^{-1}) and the amount of lipid present in the medium is the lipid yield (LY, g L^{-1}). Lipid productivity is the amount of lipid produced over time (g L^{-1} d^{-1}), a crucial measure for determining the profitability of a potential industrial production process. While highly

dependent on species, typically over 90% of the lipids that oleaginous yeasts produce are stored as triglycerides as opposed to the polar lipids (such as phospholipids or glycolipids), or other neutral lipids (such as mono- or di-acyl glycerides, free fatty acids and esters of sterols).[81, 83] These triglycerides are chemically similar to vegetable oils.[29-31] Like vegetable oils, the fatty acids that predominate in approximately descending order are oleic (18:1), palmitic (16:0), linoleic (18:2) and stearic (18:0) acids. However, the exact proprotions and lipid content vary according to species and growth conditions.[80, 84]

Most yeasts reproduce asexually by budding, in which the parent cell expands its cell wall into a bud, undergoes mitosis then separates into two cells with the daughter cell being initially smaller. By this mechanism, yeasts cells can double in number every few hours (the doubling time dependant on the species) and so the growth of oleaginous yeasts follows the growth cycle illustrated in Figure 1.4. Yeasts can also reproduce sexually by forming haploid spores when under stress, such as by having nutrient limited media.[85, 86] The specific growth rate is described by Equation 1, in which cell dry mass (X) changes in concentration over time (t) proportionally to some factors (a) such as oxygen content, substrate concentration, pH, temperature and so on, and inversely proportionate with another factor (b) such as inhibitor concentration. Accordingly, when substrate concentration is high and inhibitor concentration is low at the start of the culture (1), growth is rapid with no lag phase. As yeast cells reproduce, exponential growth occurs resulting in the substrate being consumed (3). At this point, growth ceases (5) and if inhibitor concentration increases, the death phase occurs (8).[87] During the stationary phase, if nitrogen content has been near exhausted, lipid accumulation occurs in oleaginous yeasts (see section 1.2.2).



$$Equation\ 1 \quad \frac{dX}{dt} = \frac{aX}{b}$$

Figure 1.4. Expected change in numbers of yeasts cells via the indirect measurement – optical density (filled circles) and change in specific growth rate (solid line) with time in a batch culture. (1) Lag phase, (2) acceleration phase (3) exponential (logarithmic) phase, (4) deceleration phase, (5) stationary phase (6) accelerated death phase, (7) exponential death phase, (8) death or survival phase. Adapted from El-Mansi et al.[87]

1.2.1 Species of oleaginous yeasts

The first published measurement of lipid in a yeast, *Saccharomyces cerevisiae,* was in 1878, although as a classified non-oleaginous yeast, it had a maximum lipid content of only 9%.[88] The number of species of fungi found to be oleaginous has increased vastly during the most recent spike in academic interest in the subject. In 1988, Ratledge was able to cite just eighteen species, not including *Metschnikowia pulcherrima* or *Rhodotorula glutinis* and only one strain of *Yarrowia lipolytica*, that were oleaginous.[89] However, by 2014 over eighty strains comprising twelve moulds and 74 yeasts (66 yeasts species) have been reported as having the potential to produce a lipid content of 20% or more (Table 1.7).

Culture conditions for each species vary, but the majority of reports cultured the yeasts between 25-30 °C,[23, 90-96] and a pH of between 5-6 (though five studies used a pH between 4-4.9.)[23, 97-100] In all cases either yeast extract and/or ammonium salts were used as the nitrogen source.

Non-yeast fungi

Strain name	LC (%)	Ref	Strain name	LC (%)	Ref
***Aspergillus niger* IICTGSVMLI**	23.5	101	***Mortierella alpina M6***	38.4	102
Aspergillus niger LFMB	57.4	103	***Mortierella isabellina MUCL 1430***	33.9	95
Aspergillus niger NRRL 364	41.5	103	***Mortierella ramanniana MUCL 9.235***	44.4	92
Aspergillus oryzae A-4	25.4	104	***Mortierella. isabellina ATHUM 2935***	75.0	105
Cunninghamella echinulata ATHUM 4411	39.8	106	***Mucor circinelloides 277.49***	46.0	107
Microsphaeropsis sp.	34.7	108	***Zygorhynchus moelleri MUCL 1430***	43.2	95

Yeasts

Candida aff. tenuis	56.6	109	***Metschnikowia gruessii***	34.0	110
Candida diddensiae	37.0	110	***Myxozyma melibiosi T***	23.4	84
Candida freyschussii	32.0	111	***Myxozyma udenii***	20.3	78
Candida tropicalis	21.0	110	***Phaffia rhodozyma NRRL-Y-10921***	39.7	93
Candida 107 (Pseudozyma aphidis)	37.1	112	***Rhodosporidium babjevae 05-775***	58.0	84
Cryptococcus humicola	35.5	84	***Rhodosporidium diobovatum***	50.7	113
Cryptococcus aerius	63.0	110	***Rhodosporidium paludigenum***	39.7	84
Cryptococcus aff. taibaiensis	37.4	84	***Rhodosporidium sphaerocarpum***	43.0	114
Cryptococcus albidus var. CBS 4517	46.3	115	***Rhodosporidium toruloides***	76.1	116
Cryptococcus cf. aureus	21.0	110	***Rhodosporidium toruloides 21167***	63.6	117
Cryptococcus curvatus ATCC 20509	75.0	118	***R. toruloides AS2. 1389***	69.7	119
Cryptococcus curvatus ATCC20508	48.0	120	***R. toruloides Y4***	65.4	121
Cryptococcus curvatus Ufa25	40.0	100	***Rhodotorula colostri***	26.9	84
Cryptococcus curvatus D	30.0	122	***Rhodotorula glacialis***	68.0	123
Cryptococcus curvatus UfaM3	45.6	124	**Strain name**	**LC (%)**	**Ref**
Cryptococcus laurentii UCD-FST 12-803	28.0	125	***Rhodotorula glutinis IIP-30***	66.0	23

Strain name	LC (%)	Ref	*Rhodoturola glutinis TISTR 5159*	60.4	29
Cryptococcus oeirensis	25.8	84	*Rhodotorula gracilis CFR-1*	68.0	126
Cryptococcus ramirezgomezianus	40.1	84	*Rhodotorula graminis*	52.3	127
Cryptococcus terreus	51.7	84	*Rhodoturola sp LFMB 22*	52.3	95
Cryptococcus terricola	39.0	94	*Rhodotorula minuta IIP-33*	15.0	128
Cryptococcus victoriae	22.1	129	*Rhodotorula mucilaginosa*	32.7	84
Cryptococcus wieringae	52.7	84	*Rhodoturola mucilaginosa TJY15a*	48.6	130
Cyberlindnera jadinii	22.0	78	*Rhodotorula sp LFMB 6*	22.5	95
Cyberlindnera saturnus	25.0	110	*Rhodotorula terpenoidalis*	27.0	114
Galactomyces candidus	50.0	78	*Schwanniomyces occidentalis*	23.0	110
Galactomyces pseudocandidus	28.0	114	*Sporobolomyces roseus*	38.6	131
Hannaella aff. zeae	25.6	84	*Torulaspora delbrueckii*	40.0	114
Kodamaea ohmeri	53.3	110	*Tremella encephala*	41.7	84
Kodamaea ohmeri	25.2	98	*Trichosporon coremiiforme*	37.8	132
Kurtzmaniella cleridarum T	33.3	84	*Trichosporon cutaneum*	32.0	133
Leucosporidiella creatinivora	62.0	134	*Trichosporon dermatis*	40.1	135
Lipomyces doorenjongii	72.3	136	*Trichosporon guehoae*	37.5	84
Lipomyces kockii	77.8	136	*Trigonopsis variabilis*	43.7	110
Lipomyces starkeyi AS 2.1560	54.9	91	*Wickerhamomyces ciferrii*	22.0	110
Lipomyces starkeyi ATCC 12659	29.1	137	*Yarrowia lipolytica Po1g*	58.5	138
Lipomyces starkeyi DSM 70295	72.0	91	*Yarrowia lipolytica LGAM S(7)1*	40.0	139
Lipomyces tetrasporus	66.5	110	*Yarrowia lipolytica ACA-DC 50109*	44.0	140
Lypomyces lipofer IBPhM y-693	51.5	141	*Zygolipomyces lactosus*	66.5	141
Magnusiomyces magnusii	27.0	78	*Trichosporan fermentans*	56.6	142
Metschnikowia pulcherrima	30.0	143	*Trichosporan histeridarum*	33.8	114

Table 1.7 Fungal species and, where applicable, strains reported as oleaginous, with a lipid content (LC) of 20% or more.

In terms of the mode of culture, the majority of fermentations were carried out in shake-flask batch reactions,[84, 95, 137] between 150 and 200 rpm although the greatest lipid yields are attained under fed-batch conditions (see 1.3.2, below). The greatest ever lipid content attained with non-GM yeasts was with *Lipomyces kockii* at 77.8 %, utilising 100 g L^{-1} glucose. Higher lipid contents generally lead to higher lipid yields, though the correlation is weak, especially between different carbon sources. The most studied oleaginous yeasts are *Y. lipolytica* ,[138-140, 144-152] *L. starkeyi*[91, 136, 153-155], *C. curvatus*,[120, 137, 156-162] *R. glutinis*[29, 97, 163-169] and *R. toruloides.*[90, 170-179]

1.2.2 Biochemical mechanism for lipid accumulation in oleaginous yeasts

Yeasts use triglycerides to provide a source of energy in times of stress[79, 180] and as a source of compounds that further defend against stress.[180, 181] The triglycerides may be converted to phospholipids, which, along with sterols and other lipids reside in the membranes that surround the cell or organelles. The endoplasmic reticulum is responsible for converting lipids from one form to another, in forming intracellular lipid droplets (lipid bodies) and modifying and assembling triglycerides whereas the mitochondrion are important in synthesising the building blocks for triglyceride synthesis.

Lipid accumulation can be induced in oleaginous yeasts through nitrogen starvation, where the growth medium contains an excess of carbon relative to nitrogen (ratios between 30 and 70), though lipid accumulation has been demonstrated through alternative stress mechanisms such as phosphorus limitation.[29-30] When nitrogen is limited, a four stage process occurs within the cell, that slows synthesis of protein and nucleic acid (both of which have a high nitrogen content) and instead funnels the carbon into fatty acid synthesis. In order to carry out lipid biosynthesis in nitrogen limited conditions, Ratledge and Boulton[182] discovered that oleaginous species, unlike non-oleaginous species, make use of two crucial enzymes: ATP: citrate lyase (ACL), which is present only in oleaginous species, and malic enzyme, which although is present in all fungi, only oleaginous species use it in in lipid synthesis.[182] ACL cleaves citrate into oxaloacetate and acetyl coenzyme A.

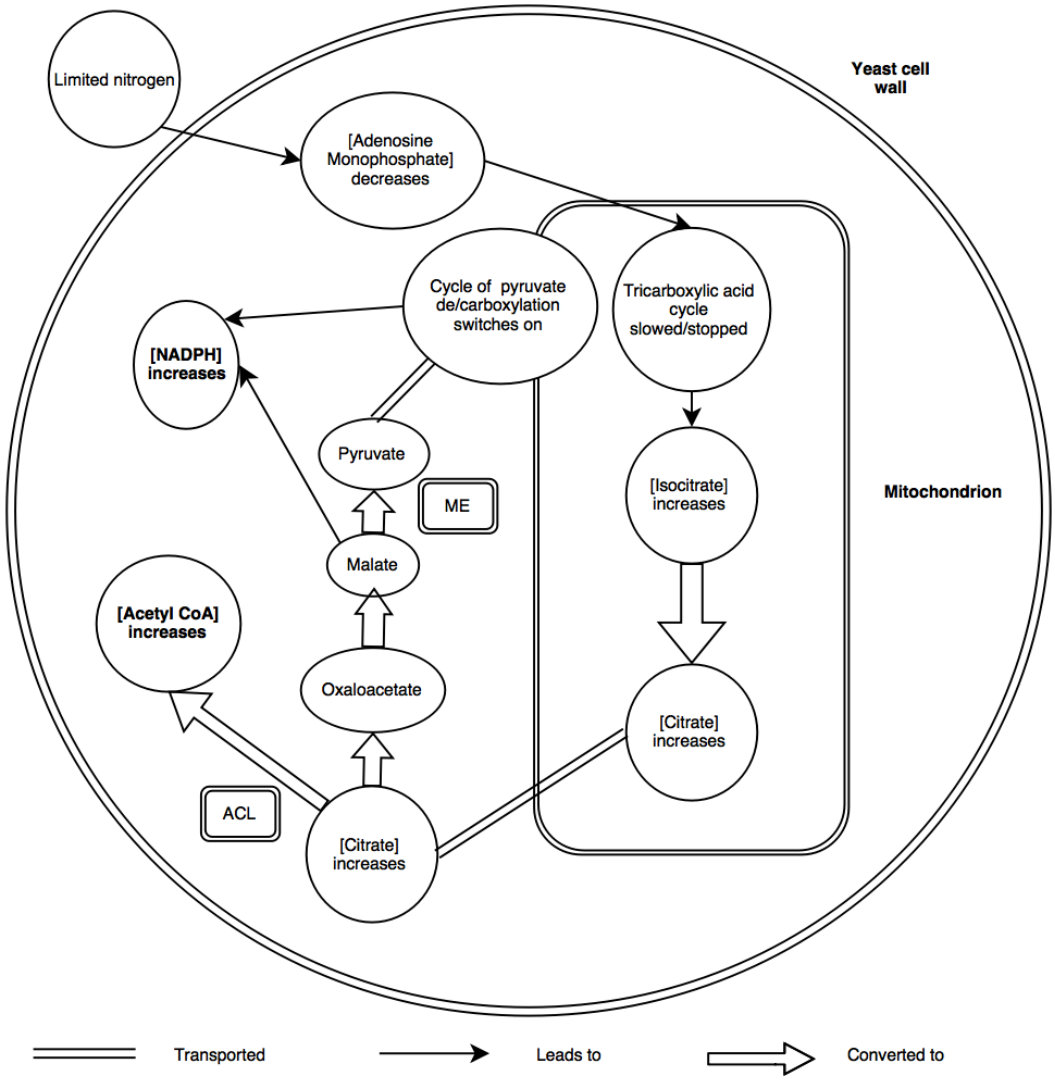


Figure 1.5 A simplified scheme of how, in oleaginous yeasts, limited nitrogen in the medium leads to an increase in the concentration of NADPH and acetyl coenzyme A within the yeasts cytosol. For clarity, only enzymes specific to oleaginous yeasts species are shown, although all chemical reactions shown are enzyme catalysed.

Malic enzyme converts malate to pyruvate, producing nicotinamide adenine dinucleotide phosphate (NADPH) as a by-product. NADPH acts as a reducing agent, and so is required to turn highly oxygenated sugars into fatty acids. In oleaginous yeasts, malic enzyme changes from one isoform to another when nitrogen is limited. The mechanism for fatty acid biosynthesis is otherwise that determined by the Nobel laureate Lynen and co-workers (Figure 1.5).[183] Transport of glucose into the cell is an active process moderated by glucose transporters.[184] Nitrogen limitation causes the cell to activate the enzyme adenosine monophosphate deaminase, which reduces the content of AMP in the cell. This means that one of the enzymes involved in the citric acid cycle (isocitrate dehydrogenase) ceases to function and the citric acid cycle, a crucial part of respiration, slows down. As isocitrate dehydrogenase usually converts isocitrate into another intermediate, isocitrate

instead builds up within the mitochondria, where it is converted to citrate by acotinase. Citrate is then transported out of the mitochondria, preventing it from taking part in the rest of the citric acid cycle.

This is where oleaginous yeasts differ from non-oleaginous yeasts,[182] as they have ACL, which splits citrate into oxaloacetate and an acetyl group, which then reacts with coenzyme A, forming acetyl CoA. The oxaloacetate is reduced to malate, then the second enzyme that is unique to oleaginous yeasts, malic enzyme, converts malate into NADPH and pyruvate. The conversion of malate to pyruvate is coupled to another cycle in mitochondria in which pyruvate is carboxylated to form oxaloacetate, transformed into malate and decarboxylated to form pyruvate, thus producing more NADPH (not shown in Figure 1.5). [185]

Acetyl CoA consists of coenzyme A attached to a two-carbon Acyl group. Acetyl CoA is converted by carboxylation with CO_2 (dissolved bicarbonate ions) to malonyl CoA, a three carbon unit. The enzyme that catalyses this step (acetyl-CoA carboxylase), uses the vitamin biotin as a co-factor to carry the CO_2 – suggesting that vitamins are an important additive to the growth medium.[110] Malonyl CoA is then converted into fatty acids via the enzyme complex; fatty acid synthase. This process requires NADPH to reduce the highly oxygenated malonyl CoA. The end-product is palmitic acid, bound to acyl carrier protein, which is, as the name suggests, then transferred back onto co-enzyme A.[78, 185]

The acyl-CoA then may be attached to glycerol (in the form of glycerol-3-phosphate) to form triglycerides, or to a phosphate group to form a phospholipid (for cell membranes). The endoplasmic reticulum is capable of elongating and desaturating the fatty acyl chains, using another form of malic enzyme and a desaturase enzyme respectively. The triglycerides then coalesce into protein-stabilised droplets, via the endoplasmic reticulum.[78, 185]

The stoichiometry of fatty acid synthesis in all eukaryotes means that the theoretical maximum efficiency of conversion from carbon source to lipid is 33%, as the generation of NADPH from malate involves the loss of a CO_2 molecule the synthesis of one mole of an eighteen carbon fatty acid requires sixteen moles NADPH. Although lipid contents can be as high as 70% this is inefficient, as the cell must also use the sugar to create cell mass and provide energy for other biochemical processes and so the optimum is closer to 40%.[50] In practice the overall conversion efficiency of sugar to lipid is around 23%.[50, 90]

1.2.3 Increasing the lipid yield in oleaginous yeasts

1.2.3.1 Culture conditions

The fatty acid profiles and lipid content usually but not always, vary depending on the species and culture conditions,[79, 84, 119, 186] which include temperature, fermentation time, the composition of the growth medium, aeration and pH.[78, 79]

A crucial component in initiating lipid accumulation is the ratio of carbon to nitrogen available to the yeast. As nitrogen is essential for the synthesis of proteins and nucleotides, nitrogen limitation may limit the dry cell mass as well as increase the lipid content. This often results in a low overall lipid yield (in terms of amount lipid per litre of medium). This was the case in a study by Boundy-Mills *et al.*[84] in which 69 yeasts strains were grown in identical media and conditions, aside from the presence or absence of nitrogen. Of the 56 species, 27 had an improved lipid yield in the presence of nitrogen, and 29 performed better under nitrogen-limited conditions. Of the ascomycetes (which includes *Metschnikowia pulcherrima* and *S. cerevisiae* although these were not included in this study) only two out of thirteen species yielded more lipid under nitrogen limited conditions. The basidiomycetes were more suited to increasing the lipid yield by decreasing the nitrogen content, yet this was not always the case. A balance must therefore be struck between a high enough concentration of nitrogen to allow the yeasts to reproduce, yet not so much as to prevent the initiation of lipid accumulation. Experimenting with the carbon to nitrogen ratio has to be done on a strain-by-strain basis, but the principle behind most studies is to have enough nitrogen to enable the exponential phase, which then is consumed entirely once the stationary phase is reached, yet there is still an excess of carbon. Such experiments are commonplace in the oleaginous yeasts literature.[133, 142, 173]

The selection of the nitrogen source is also important. All yeasts species can utilise ammonium,[78] but this is energy and carbon intensive to manufacture. Some oleaginous yeasts can utilise nitrate,[129] and oleaginous species have been shown to consume waste nitrogen sources, most commonly yeast extract (though this is costly) and tomato waste hydrolysate.[106] Organic nitrogen in the form of yeast extract and peptone has been shown to yield higher cell dry mass and higher lipid content, whereas inorganic nitrogen (such as ammonium salts) increases mostly cell dry mass, suggesting that a mixture of the two might be the most economically advantageous.[160] A few examples exist of oleaginous yeast growth on wastewaters, such as *L. starkeyi* on sewage sludge,[91] yielding 6.7 g L^{-1} lipid (72.3 % lipid content).

One of the simplest ways to increase the lipid content of oleaginous yeasts is to increase the amount of sugar or other carbon source available to them.[136] Having a high sugar

concentration not only increases the number of yeasts cells that are able to grow, but it also means they convert this excess carbon into lipid.[123, 136] This method comes with the caveat that increased sugar concentrations also lead to elevated osmotic pressures, that can limit growth substantially the yeasts as intracellular water escapes through the cell membrane,[160] and so an optimum sugar concentration must be found.

The majority of studies into oleaginous yeasts have used glucose as a carbon source.[23, 78, 79] Other sugars generally yield lower lipid yields.[23, 78, 79] More unusual substrates have been derived from non-lignocellulosic wastes such as whey,[100] starch, [117,130] Jerusalem artichoke tuber extract[95], ethanol[98] and methanol.[98] When a variety of carbon sources have been examined under otherwise identical conditions, it has been found that varying the type of sugar varies both the lipid content[136] and fatty acid profile.[84] For example, when grown on cellobiose (70 g L^{-1}) alone, *L. starkeyi* attained an impressive dry cell mass of 27.9 g L^{-1} and lipid content of 50% giving a lipid yield of 13.95 g L^{-1}, whereas glucose at the same concentration (by mass if not by moles) yielded 12.3 g L^{-1} lipid.[155]

When multiple sugars including glucose are present, yeasts will preferentially consume glucose. Only when the glucose in consumed will yeasts produce the cellular machinery to begin to consume other sugars (diauxic growth). The glucose repression mechanism involves the presence of glucose (and also galactose and maltose) inhibiting the transcription of genes, which encode enzymes that catabolise non-glucose sugars. If these enzymes are already present, they are degraded faster in the presence of glucose[184, 187] When the concentration of glucose is lower, as is the case during the stationary phase of fermentation, glucose repression ends and the genes that catabolise other sugars are instead promoted. The significance of this in practical terms is that fermentations on lignocellulose hydrolysates, which are a mixture of sugars including glucose, can be slower with yeasts that experience significant diauxic growth than those performed on pure sugars. Glucose repression therefore limits the lipid productivity of the hypothetical industrial process.[184]

For certain species, glucose repression can be overcome by tuning the sugar composition of the growth medium so that there is a large quantity of non-glucose sugars. Interestingly, when *L. starkeyi* was cultured on xylose combined with cellobiose (a disaccharide of glucose) but no glucose, the yeasts simultaneously used both sugars. This indicates that *L. starkeyi* either contains or excretes a β -glucosidase enzyme that can break β-1,4-glycosidic bonds, which then turns cellobiose into glucose and the glucose pathways are followed. An experiment with low glucose concentrations, and high cellobiose and xylose concentrations, yielded a cell dry mass 25.5 g L^{-1}, of which 52% was lipid (13.26 g L^{-1} lipid) in 108 hours. These results are similar to those found when the

yeasts was grown on single sugar sources. The authors note that such a feedstock composition could be obtained from lignocellulose hydrolysed by a cellulose mixture without β –glucosidase, saving on hydrolysis costs.[155]

Fermentation is an exothermic process and most oleaginous yeasts require temperatures between 25-30 °C. Many strains of oleaginous yeasts grow optimally in a small temperature range and this temperature must be carefully controlled for optimal growth. Temperature is a key factor in lipid yields and can alter the fatty acid composition of cell membranes.[188] In one study, 26 species of oleaginous yeasts were shown to produce higher lipid contents at 4 °C or 18 °C than at 30 °C.[134] While temperature does have an effect on lipid composition, this is highly species dependent. However, the fatty acid composition of the cell membrane tends towards higher proportions of polyunsaturated compounds at lower temperatures, though not in all species studied, and not for all unsaturated fatty acids. For example, Shaw[186] varied the temperature and time of cultivation of the moulds *Rhizopus arrhizus* and *Cunninghamella blakesleeana* in an attempt to increase the proportion of gamma-linolenic acid. They found that the overall degree of saturation did not change with temperature, although the fatty acid profile did. This is in contradiction to the hypothesis that fungi grown at lower temperature will have a lower degree of saturation in order to maintain the fluidity of the cell membrane as unsaturated fatty acids have lower freezing points.[188]

Commonly oleaginous yeast cultures are held for at least 5 days before the maximum lipid content is attained, though three, four and six days are not uncommon.[23, 79] Once the yeasts have reached the stationary phase and all nitrogen is consumed then the lipid accumulation step can take place. In most yeasts if the culture is held for longer, then the yeast can reduce the lipid content, as the lipids are used as an energy source.[180, 189]

Lipid accumulation also requires oxygen, in the order of 1–3 moles O_2 per kg cell dry mass per hour. For almost all oleaginous yeasts there is a direct correlation between oxygen content of the media and lipid content.[180, 190] At the laboratory scale, higher lipid contents are achieved when baffled flasks with foam stoppers are used than smooth airtight tubes.[125] At an industrial scale this need for aeration is a large component of the capital costs of a plant, as air compressors are expensive.[191] Airlift and bubble fermenters, where the mixing is provided by air flow from the base of the reactor, have been suggested by Davies[192] as a potential solution to the aeration issue, and these have been demonstrated as a suitable bioreactor for *C. curvatus*.

1.2.3.2 Genetic modification of oleaginous yeasts

While not as developed as bacterial systems or *S. cerevisiae*, some work on and genetic engineering of oleaginous yeasts, predominantly traits such as the overexpression of lipid, have been attempted.[79, 144, 193, 194] For example, *Yarrowia lipolytica* has been modified through multiple gene modifications to overexpress lipid. This led to an impressive 20 g L^{-1} dry cell mass with a lipid content between 60-88%. Consequently, the lipid yield reached 15 g L^{-1} in 140 hours.[143]

However, in a European context, and especially in the food and consumer products in which palm oil is found, this is not necessarily beneficial due to public and legislative opposition to genetically modified organisms (see also 1.2.4.1 below). In the short term at least it seems likely that a palm oil substitute will have to come from a non-GM based system.[195]

1.2.4 Industrial production of fungal oils

The industrial production of lipid from yeasts was first attempted during World War One. After concluding that aeration was a problem in generating reasonable lipid yields, Lindner *et al.* developed pilot scale methods in both a tray and a solid-state fermentation on a straw matrix to culture *Trichosporon pullulan.*[78, 88] However, this method led to severe contamination, it was an inefficient use of space while the extraction of the oil made the cost too high – resulting in the oil filled yeasts produced being used as fuel for horses rather than combustion engines.[88] An estimated 340 tonnes of fungal oil were produced in 2003.[196] Of the processes that use fungi to produce oil by de-novo synthesis that have been successfully commercialised, all but one use moulds rather than yeasts, and all are rich in high-value polyunsaturated fatty acids.

1.2.4.1 Polyunsaturated fatty acids as food additives

The earliest commercial process attempted to make oils rich in the fatty acid γ-linolenic acid (all-cis18:3 [6,9,12). This component of evening primrose oil had many claims made about its health benefits related to it being a precursor to several hormones.[197] The two moulds (*Rhizopus arrhizus* and *Cunninghamella blakesleeana*) were examined by Shaw[186] at Unilever. Both moulds yielded significantly different total lipid yields, as well as yields of the desired fatty acid under the same conditions. Frustratingly for their aims, the species that gave the greatest overall lipid yield (4.2 g L^{-1} vs. 0.4 g L^{-1}) had only half the γ linolenic acid content.[186] This variability demonstrates the importance of species selection and process conditions, which do not necessarily coalesce into one obvious set point.

The process was finally commercialised by the British firm J. & E. Sturge Ltd, assisted by the technical expertise of Ratledge. They ran the fermentation at a 220-m^3 scale using the

mould *Mucor (javinicus) circinelloides*, and the same product was made by Idemitsu Co. Inc. (Japan) using *Mortierella isabellina*. The J. & E. Sturge process yielded a mould with a 20-25% lipid content, 15-18% of which was the target fatty acid. Although Idemitsu still manufacture γ-linolenic acid via fermentation,[198] J. & E. Sturge process is no longer operational presumably for commercial reasons. The medical claims surrounding γ-linolenic acid have widely been debunked,[199] and as a result in 2003, UK regulators prevented the marketing of evening primrose oil as a treatment for eczema.[200]

The most commercially successful production of oil from fungi is the production by the chemical company DSM (and others) of the omega-6- fatty acid arachidonic acid (C20:4) for use in infant formula milk and health supplements.[196] In the DSM process, the mould *Mortierella alpina* is cultured at up to 100 m^3 scale via a series of seed fermenters fed with glucose supplemented with nitrogen salts. The oil is then extracted from the spray-dried biomass using hexane and is thought to contain antioxidants that help to preserve the product. The high value of arachidonic acid compensates for the costly feedstock, sterilisation and in particular the air compressors required for sufficient mass transfer of oxygen to cells when carrying out the aerobic synthesis of unsaturated lipids.[196]

More recently, DuPont have begun using genetically engineered strains of *Y. lipolytica* in which 56.6% of the oil produced is eicosapentaenoic acid.[145, 201, 202] In the present climate, a genetically modified food source would be difficult to sell (especially in the EU), and this and the high price of the oil may have led DuPont withdraw their nutritional supplement product in 2013.[203] For lower value care product emulsions, genetic modification, pure glucose, sterilisation and air compressors may prove be too costly.[195] Not separating the oil from the yeasts is also perhaps more commercially viable – DuPont still sell yeasts rich in eicosapentaenoic acid to Verlasso® to feed farmed salmon, on the grounds that this reduces the burden of salmon farming on fish stocks.[203, 204]

1.2.4.2 Manufacture of a Cocoa butter replacement

Cocoa butter is a valuable fat used in chocolate and personal care products that during the mid-1980s became sufficiently expensive (US $8000 per tonne) that some researchers attempted to use oleaginous yeast to create an oil with equivalent properties.[195] The thick texture of this oil relative to other vegetable oils arises from its high content of stearic acid (18:0). Oleaginous yeasts typically form stearic acid during fatty acid synthesis, then it is desaturated into oleic acid (18:1) by Δ^9-desaturase, resulting in fatty acid compositions with more oleic acid than stearic acid, whereas the proportions in cocoa butter are approximately equal. Accordingly, one of the more successful attempts was by Smit[196] who deleted the gene that codes for Δ^9-desaturase from C*ryptococcus. curvatus D* affecting an increase in stearic acid content from %12 to 50%. However, the excess of linolenic acid

(18:2) and insufficiency of palmitic acid (16:0) meant that the product was still too dissimilar from cocoa butter to be used as a substitute.[88, 196]

Despite imperfections, one of the largest scale oleaginous yeasts fermentations yielded a product that had a comparable manufacturing cost to the sale price of palm oil. In this case, the stearic acid content was increased by utilising the oxygen dependency of Δ^9-desaturase. Growing *C. curvatus* in low oxygen conditions increased the yield of stearic acid and decreased the yield of other unsaturated acids. An acceptable cocoa butter substitute was therefore obtained from waste lactose from the cheese industry.[195] However, despite the near-zero cost of this feedstock, the product (manufactured for US $800-1000 per tonne and saleable for $2000 per tonne) was insufficiently profitable compared to genuine cocoa butter, which by the time process development was complete cost $3500 per tonne.[192, 196] The components of this process, which contributed most significantly to this cost were not elucidated, however they were calculated using a 8200 L fermenter which had limited process controls and required the inoculum to be produced off site. This change to the process coincided with a slower rate of lactose uptake and a reduction in lipid content cf. the 500 L pilot process (39% to 23%). Although continuous processing gave the highest productivity, the whey feedstock was of inconsistent quality and availability and so the 88% less productive batch process was selected. The oil also required separation from the yeasts, which they achieved using nozzle disc separators, disruption of the cells via a bead mill then extraction with ethanol and hexane.

1.2.4.3 To improve the efficiency of biodiesel production

In 1993, Ratledge wrote of the consensus in scientific opinion - oils from yeasts were not able to replace the functions of oils from animals or plants.[88] The costs of fermentation technology were higher than that of agriculture, and of single cell protein (used as animal food) production. Important factors in the cost were the feedstock and extraction of the oil. It has been estimated that if glucose were used it would contribute $2.07 kg^{-1} out of the $5.48 kg^{-1} manufacturing cost of the oil.[191] Ratledge concluded that oleaginous yeasts could only be economically viable for expensive polyunsaturated fatty acids.[88] He later added the caveat that if a large quantity of cheap substrate was available, in particular as a way to upgrade waste materials, that yeasts could be potentially useful.[89] Since 1990, motivations from sustainability,[13] energy price[205] and consequent governement pressure[206] have led to a re-assessment of Ratledge's view. Now the most commonly cited potential use is for biodiesel.[31, 78, 191] In addition, a waste product from the biodiesel manufacturing process, glycerol, can be transformed into triglycerides by oleaginous yeasts, leading to increased biodiesel yields and lower waste disposal costs.

Species	X (g L^{-1})	LC (%)	LY (g L^{-1})	T (°C)	t (h)	Glycerol source[a]	Culture mode	Ref.
Candida aff. tenuis	16.1	56.6	9.1	28	192	Waste	Batch	109
Cryptococcus curvatus	91.0	33.3	30.3	28	75	Pure	Surer®	161
Cryptococcus curvatus ATCC 20509	31.2	44.6	13.9	28	288	Waste	One stage, fed-batch	207
Cryptococcus curvatus ATCC 20509	32.9	52.9	17.4	28	288	Waste	Two stage, fed batch	207
Cryptococcus curvatus ATCC20508	69.0	48.0	33.1	28	134	Waste	Fed-batch	120
Rhodosporidium babjevae 05-775	9.4	24.1	2.3	30	120	Waste	Batch	113
Rhodosporidium diobovatum	13.6	50.7	6.9	30	120	Waste	Batch (Bioreactor)	113
Rhodosporidium toruloides AS2. 1389	26.7	69.7	18.6	30	160	Waste	Batch	175
Rhodotorula glutinis ATTC 204091	24.4	36.9	9.0	35	48	Pure	Batch	165
Rhodoturola glutinis TISTR 5159	10.1	60.4	6.1	30	72	Waste	Fed-batch	29
Yarrowia lipolytica LGAM S(7)1	4.6	19.6	0.9	28	50	Waste	Repeated batch culture	149
Yarrowia lipolytica Po1g	6.8	42.6	2.9	28	271	Waste	Batch	95

Table 1.8. Oleaginous yeast strains that attained a lipid content of ≥ 20% when grown on [a] unrefined waste glycerol from production of biodiesel or on purified glycerol. Surer ® is a brand of fermenter. Initial pH for all results was 5-6. X = dry cell mass, LC = lipid content as % wt/wt of dry cell mass, LY = lipid yield per litre of medium, T = temperature, t = fermentation time at which maximum lipid content was attained.

It has been demonstrated that oleaginous yeasts can grow about as well on the crude waste glycerol that emerges from the biodiesel plant contaminated with over 5% methanol and sodium as it can on purified forms.[113, 161] Glycerol has therefore been studied as a potential feedstock (Table 1.8)..

For instance, cultivation of *Candida aff. tenuis* on raw glycerol produced an oil with a similar fatty acid profile to soybean oil (Table 1.8). Duarte *et al.* estimated that 66 m^3 of glycerol based medium would yield the same amount of soybean oil produced by one hectare of farmland in a year in just ten days.[96] This is particularly relevant given that the study was based in Brazil, where soybean farmland is frequently created from the destruction of native forest, as per palm oil in South East Asia. Given that this yeast yielded 9 g L^{-1} the higher yields from fed batch cultures may be even more efficient

1.3 Valorisation of lignocellulose by oleaginous yeasts

The vast majority of progress in microbially valorising lignocellulose is for production of lignocellulosic ethanol using *S. cerevisiae*, though the feasibility of deriving chemical products from lignocellulose has also been demonstrated industrially with over 40 biorefineries operating to date.[208-212] For example, Borregaard, has produced bioethanol by sulfuric acid pretreatment of eucalyptus wood, followed by enzymatic hydrolysis, and fermentation with *S. cerevisiae* since 1938.[208] A range of co-products including products that require chemical upgrading help to increase the profitability of the plant. Another more recent example is the Inbicon pilot plant,[210] brought online in 2012, which uses steam pre-treatment (with small quantities of acetic acid) at 180-200 °C for 10-20 min to solubilise the hemicellulose and disrupt the lignin.[212] After separating the solid and liquid fractions and adjusting the pH, cellulases are added at high loadings (250 kg m^{-3}) to complete the hydrolysis.[210] This process uses the yeast *S. cerevisiae* to ferment glucose from cellulose to ethanol, in a continuous fermentation. It seems likely that these processes can be adapted simply to oleaginous yeast fermentations for the production of lipid from lignocellulose.

1.3.1 Hydrolysis of lignocellulose

Compared to the starch component of plants, lignocellulose is resilient to chemical and enzymatic hydrolysis of its glycosidic bonds (see section 1.1.4 above). Lignin is generally impervious to water and these enzymes, and so hydrolysing the intact material is far from trivial. Generating a sugar-rich lignocellulose hydrolysate as cheaply and sustainably as possible is a crucial issue that can be addressed partly by species selection and partly by process design.[213, 214]

The general process involves three steps.

- Firstly, the lignocellulose is mechanically broken down into smaller particles by using a cutting machine,[210] a ball mill[215] or a chipper.[34]
- Secondly, a process to hydrolyse the glycosidic links in the hemicellulose and cellulose is carried out. The hydrolysis step has been extensively studied and most often methods involve a pretreatment step, followed by neutralisation (where necessary) and enzymatic hydrolysis,[34, 216-222] and less often involve a more intense version of one of the pretreatment methods that negates the need for enzymes.
- Finally, the mixture of monomeric sugars and inhibitors produced is modified to the conditions under which the fermentation will take place. Modifications usually include separating the mixture of solids (mostly lignin and un-hydrolysed biomass) from the liquid phase that contains the sugars, detoxification to remove toxic compounds and adjusting the pH to one suitable for microorganism growth.

Hydrolysis by enzymes is only effective if one of a myriad of pre-treatment processes is used to allow accessibility to the cellulosic chains by removing the lignin, hydrolysing some of the hemicellulose, and reducing the degree of crystallinity and the degree of polymerisation of the cellulose. This allows the enzymes to access the newly exposed cellulose fibrils and leads to an increased yield.[34] One of the benefits of enzymatic hydrolysis is that it does not produce growth inhibiting compounds (see section 1.3.3.2, below), however, many of these pretreatment methods do.

A common pre-treatment method is steam explosion, where water and biomass are heated (>230 °C) under pressure (550 psi) for up to two minutes, then the pressure is suddenly reduced. The rapid reduction in pressure leads to the physical separation of the cellulose fibres. This can degrade up to two thirds of the hemicellulose and degrades the cellulose fibres into smaller fragments. This process leads to the production of inhibitory compounds.[223]

The ammonia fibre explosion (AFEX) process employs a similar method using alkaline ammonia to catalyse hemicellulose and cellulose hydrolysis. An exemplary AFEX process optimised for switchgrass uses 1.1 eq. ammonia to dry lignocellulose at 100 °C for 5 min, followed by enzymatic hydrolysis to give a 93% yield of glucose. Although the ammonia can be recycled, thus limiting its environmental impacts, in the case of oleaginous yeasts this would increase the nitrogen content of the hydrolysate to unacceptably high levels.[224]

Alkalis (most commonly sodium hydroxide or lime) are particularly effective at delignification, and typically use 1.5% sodium hydroxide at 120 °C over 2 hours in an autoclave. The enzymes would be denatured by the high pH, and so the solids must be

washed extensively before neutralisation to avoid a high concentration of salt, which would also denature the cellulase enzymes.[225, 226]

Enzymatic hydrolysis is the most common and industrially applicable hydrolysis method. This employs cellulases to catalyse the hydrolysis of the cellulose. Cellulases fit into two categories based on their modes of action. Endoglucanases cleave β-1,4-glycosidic bonds at random intervals in the middle of a cellulase chain to create shorter chains of cellulose known as oligosaccharides. Endoglucanases then remove two glucose units (cellobiose) at a time from either the reducing or the non-reducing ends of the cellulose chains. β -glucosidase then hydrolyses cellobiose into glucose. All or some of these enzymes may be added to pre-treated lignocellulose, as well as xylanases. Endo- and exo-glucanases are usually manufactured from the fungus *Trichoderma reesei*, whereas β-glucosidases come from *Aspergillus niger*.[227, 228] Although enzymatic hydrolysis is effective at converting the majority of cellulose to glucose, enzymes have significant penalties in terms of cost, CO_2 emissions, energy requirements and reuse.[59, 191, 210, 224, 229]

A one-step process without the need for additional enzymes would have many benefits including potentially reducing the capital costs of a process. Lignocellulose hydrolysis can be accomplished abiotically. One of the earliest methods of lignocellulose hydrolysis employed concentrated (10-30%) acid at 50 °C, 1 atm. This takes between hours and days, but gives better conversion of cellulose to glucose than dilute acid hydrolysis.[215] On a lab scale trifluoroacetic acid is used,[230] however this is too expensive at larger scales and so sulfuric acid is generally used in scaled-up processes. Sulfuric acid is toxic, corrosive, requires energy intensive recovery processes and is non-renewable.[34]

Dilute acid hydrolysis employs a high temperature (160-230 °C) and moderate pressures (~10 atm), with 2-5% concentration of acid. This leads to comparatively low glucose yields and inhibitory by-products, which is why it is also used as a pretreatment step before enzymatic hydrolysis. The (US) National Renewable Energy Laboratory (NREL) has developed conditions (0.7% H_2SO_4, 210 °C) for extremely low acid concentrations and established that this would lead to the cheapest ethanol.[231-233] Cellulose hydrolysis occurs more rapidly and to a greater extent under more intense conditions (acid concentrations above 0.7% w/w, temperature ≥210 °C), because these conditions break the hydrogen bonds between cellulose fibrils, and so disrupt the crystallinity allowing access to the glycosidic bonds. The mechanism of this process (Figure 1.6) involves the production of soluble oligosaccharides with a degree of polymerisation less than 10, which are then hydrolysed into monomeric glucose. The glucose sometimes forms a complex with solubilised lignin.

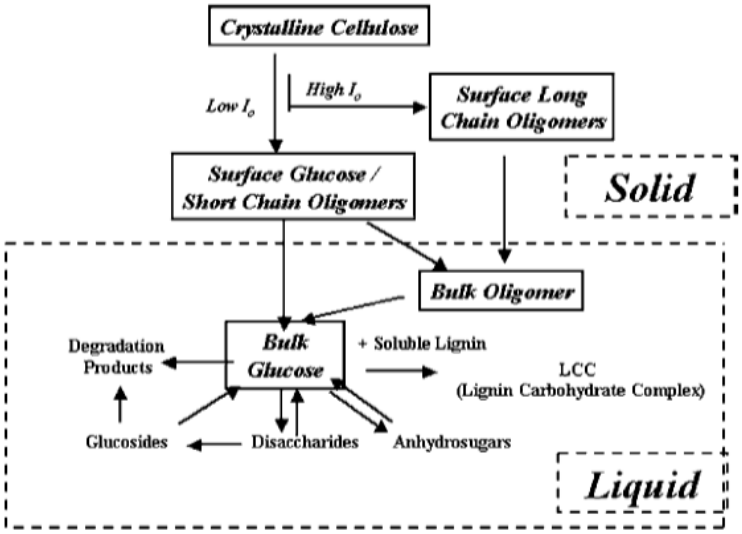


Figure 1.6. Model of processes occurring during acid hydrolysis of lignocellulose under conditions with a temperature above (high I_0) and below (low I_0) 210 °C and 0.07% acid [231]

Another method is hydrothermal, involving the application of superheated water to lignocellulose in either flow or batch processes. This can also be used as either hydrolysis method in its own right or a pretreatment step. In a recent review, Zhao *et al.*[234] defined three broad categories of research in this area:

- Temperatures over 374.3 °C and a high enough pressure (>221 MPa) to cause the water to become supercritical. This has the benefit of completely hydrolysing lignocellulose in a few seconds once the intense reaction conditions are reached. This method works through the H^+ ions found in supercritical water, which allows it to break the crystalline structure effectively.[235]
- Temperatures between 300-374 °C, which is pressurised but below the critical point so the water is in the liquid phase. The lower intensity of this process cf. subcritical water is reflected in less rapid and complete hydrolysis and lower rates of glucose decomposition (66% of cellulose if hydrolysed in 4.8 seconds at 335 °C),[236] however this intense process has high energy, capital and environmental costs.[234]
- Low temperature (150-300 °C) water is applied to the lignocellulose for approximately 15 minutes.[34] This solubilises some 4-22% cellulose, 35-65% lignin and 100% hemicellulose.

The hydrothermal process leads to the production of inhibitory compounds, especially under the most intense conditions, as monosaccharides are more rapidly broken down

into inhibitors (see section 1.3.3.2). One of the major advantages of this process is that it does not require the biomass to be physically pre-treated, as it concurrently degrades the biomass into smaller fragments. For example, Allen *et al.* found that hot (190-230 °C), pressurised water was capable of hydrolysing 80% of hemicellulose but solubilising only 10% of the cellulose in sugar bagasse that had not been physically pre-treated in under 4 minutes. [237]

1.3.2 Process configurations

There are three configurations for the hydrolysis and fermentation of lignocellulose – separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF) and consolidated bioprocessing (CBP).

1.3.2.1 Separate hydrolysis and fermentation (SHF)

In separate saccharification and fermentation, the production of enzymes, the hydrolysis step and the fermentation are carried out in separate vessels. As this replicates a classic batch process in which feedstock addition and product removal are discontinuous, the process efficiency can be increased in the same ways - by using either a continuous or fed-batch process.

Continuous fermentation, known to produce the greatest productivity in lignocellulose to ethanol fermentation,[210, 238] appears to produce high lipid productivities in oleaginous yeasts compared to batch processes.[100, 112, 122, 139, 141, 173, 178] For example, Ykema *et al.*[100] attained a lipid productivity of nearly 24 g L^{-1} d^{-1} in a continuous culture in which the feedstock whey was added and broth removed simultaneously.

The largest lipid yields from oleaginous yeasts have been achieved under fed-batch conditions,[90, 121, 178, 239] however this has only been attempted with model compounds and fructose-rich inulin hydrolysate, not lignocellulose. Fed-batch cultures are thought to be so successful as they resolve the problem of needing both as large a sugar input as possible and not having so high a sugar concentration that osmotic pressure inhibits the growth of the yeasts (see 1.2.3.1). In a fed batch process, the growth medium initially contains as high a sugar concentration as possible without leading to growth inhibition, then once the sugar is partially consumed additional portions of concentrated sugar solution are added. In a recent example, Zhang *et al.*[160] compared the lipid content and dry cell mass produced when *C. curvatus O3* was cultivated in shake flasks compared to a 3 L fed batch culture using glucose as the carbon source. In shake flasks (batch), the dry cell mass and lipid content reached 51.8 g L^{-1} and 65%, respectively whereas fed-batch culture in a 30 L

stirred tank fermenter produced a dry cell mass of 104 g L^{-1} a lipid content of 82% and lipid productivity rate of up to and 0.47 g L^{-1} h^{-1}, respectively.

1.3.2.2 Simultaneous saccharification and fermentation (SSF)

Another way to achieve greater lipid productivity is by simultaneous saccharification and fermentation in which the enzymes and yeasts are added simultaneously to pretreated lignocellulose. Simultaneous saccharification and fermentation has advantages over separate hydrolysis and fermentation as glucose and cellobiose inhibit the action of cellulases, and so as the yeasts reduce the concentration of these sugars the cellulases become more effective.[157] This has been sometimes been shown to improve the ethanol yield[240-242] compared to SHF and even if the ethanol yield is lower, the productivity is still improved. *C. curvatus* shows a similar improvement in lipid productivity for SSF (4.69 g L^{-1} d^{-1}) over SHF (3.75 g L^{-1} d^{-1}) from corn stover.

1.3.2.3 Consolidated bioprocessing

Consolidated bioprocessing is the production of enzymes, saccharification and fermentation by one microorganism in a single vessel. This is thought to save on capital costs as fewer, smaller vessels would be needed and also on the costs of raw materials, labour and enzyme production.[243, 244] The rate of product formation should also be higher. The requirements of the ideal ethanol-producing microorganism suggested by Hasunuma and Kondo[243] are the ability to produce cellulases, hemicellulases and β-glucosidases capable of hydrolysing pretreated lignocellulose into monosaccharides, efficient ethanol production in terms of concentration, yield and productivity from both pentoses and hexoses. Also important are tolerance to ethanol and other inhibitors, and robustness to stressful conditions such as low pH, high temperature, low nutrition and fluctuating conditions.[243, 245-249]

As well as moulds that excrete cellulases and xylanases onto lignocellulose,[104, 250] some yeasts are capable of producing enzymes that hydrolyse either oligocelluloses or oligoxyloses.[251, 252] One such yeast is *Scheffersomyces stipitis*, an ethanologenic yeast, that produces cellulases, hemicellulases and a β-glucosidase that allow it to thrive on decaying wood.[252] *C. curvatus* is believed to transport the oligosaccharides into the cell through endocytosis and break their β-glycosidic bonds using aryl-β-glucosidase.[157] The usefulness of this effect is demonstrated by the amount of corn stover converted to lipid increasing by 12% when no β-glucosidase or nutrients are added in SSF compared to an SHF process with nutrients and β-glucosidase. A configuration that combines continuous processing with consolidated bioprocessing (in this case to ethanol by *Clostridium thermocellum*, a bacterium that also produces cellulases) has been developed by Lynd *et*

al.[253] Although this method produces lower yields of ethanol, it reduces costs substantially, and with further development could offer an inexpensive method of producing fuels and chemicals.

1.3.3 Challenges in the fermentation of lignocellulosic hydrolysates

There are three key challenges in producing suitable products from the fermentation of lignocellulosic hydrolysate that can be addressed through species selection and process design. These are the ability to use all the sugars present in the feedstock, to increase the tolerance to inhibitors produced on depolymerisation and to limit the effect of contaminating species, allowing for lower sterility and therefore lower processing costs.

1.3.3.1 Increasing the range of sugars catabolised

Wild-type *S. cerevisiae* can only metabolise hexoses, and not pentoses or oligosaccharides meaning a significant proportion of the sugars found in lignocellulose are wasted. A yeast strain that can grow on a wider range of sugars would therefore be advantageous, as in addition to improving the yield this versatility would allow it to grow on any local biomass source, and at any time of the year.[254] Generally oleaginous yeasts can grow on a wide range of carbon sources, including pentoses[110, 143] and glycerol[95, 255]. Boundy-Mills *et al.*[110] recently screened 48 oleaginous yeasts strains for growth (but not lipid content or profile) on glucose, xylose, D- and L-arabinose, mannose, galactose, cellobiose, sucrose, galacturonic acid (a hydrolysate of pectin) and glycerol, all at a very low (0.5 g L^{-1}) concentration of sugar. Of these 48 strains, all grew on glucose and mannose, and the majority grew on xylose (46), cellobiose (45), sucrose (43), galactose (41), sucrose (43) and L-arabinose (37). Fewer strains of yeasts were able to catabolise D-arabinose (31), glycerol (30), galacturonic acid (26) or rhamnose (25). The worst performers were *Trigonopsis variabilis*, *Wickerhamomyces ciferrii* and most notably *Yarrowia lipolytica* – which was only capable of growing on glucose, mannose and glycerol. The best performers, capable of growing on every substrate tested, were *Cryptococcus cf. aureus*, *Cryptococcus humicola*, *Trichosporon coremiiforme*, and *Trichosporon dermatis,* though many others grew almost as well on these low-sugar conditions. Had the sugar concentrations been greater, as is perhaps the case in lignocellulose hydrolysates, more strains may have grown.

The inability to catabolise a sugar can be overcome by producing recombinant strains that express genes from xylose utilising yeasts such as *Scheffersomyces stipitis* [254, Jeffries, 2006 #356, 256-265] and adding cellulases and β-glucosidases from fungi such as Aspergillus aculeatus.[254, 266-269] Recombinant *S. cerevisiae* with both modifications has been shown to

increase the yield of ethanol from 31% to 41% when grown on wood chip hydrolysate, compared to a theoretical maximum of 51%.[254] Such modified strains are proposed to be particularly effective for consolidated bioprocessing (see 1.3.2.3).

1.3.3.2 Increasing inhibitor tolerance

Under the conditions required for acid hydrolysis or pretreatment, hydrothermal, ammonia or steam explosion the sugars produced are degraded into a range of compounds that inhibit the growth of microorganisms (Figure 1.7) The amount and type of inhibitors varies depending on the biomass source and hydrolysis conditions (Table 1.9).[35, 270-273]

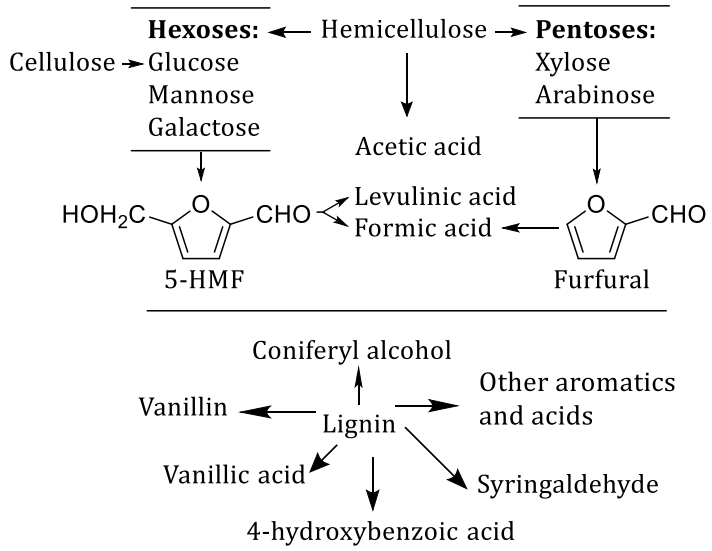


Figure 1.7 Decomposition pathways of monomeric sugars and lignin during acid pretreatment/hydrolysis. The furfuraldehydes (furfural and 5-hydroxymethyl furfural (5-HMF)) are produced from the dehydration of pentoses and hexoses, respectively. Acetic acid is produced by the hydrolysis of acetyl groups in hemicellulose, whereas the other acids arise from the acid catalysed degradation of furfurals. Lignin also partially breaks down into a range of phenolic compounds with alcohol, acid and aldehyde groups. [270, 272]

These compounds are well known to be toxic to microorganisms such as the yeasts used in bioethanol production including most strains of *S. cerevisiae*[137, 209, 271, 274-276] and *Pichia stiptis*[277] and in oleaginous yeasts.[110, 137, 164, 278] For example, *Pichia stiptis* could tolerate 0.5 g L^{-1} furfural with no negative effects, but 2 g L^{-1} halted almost all growth.[277] The oleaginous yeast *Rhodosporidium toruloides* has its growth reduced by 45% in the presence of just 0.09 g L^{-1} furfural.[179]

Lignocellulosic material	Hydrolysis conditions	Released sugars (g L^{-1})	Inhibitors profile (g L^{-1})
Sugarcane bagasse	2.5 % (v/v) HCl, 140 °C, 30 min, S: L = 1:10	Total reducing sugars, 30.29	Furans, 1.89; phenolics, 2.75; acetic acid, 5.45
Wheat straw	1.85 % (w/v) H_2SO_4, 90 °C, 18 h, S: L = 1:20	Xylose, 12.80 ± 0.25; d-glucose, 1.70 ± 0.30; l-arabinose, 2.60 ± 0.21	Furfural, 0.15 ± 0.02; acetic acid, 2.70 ± 0.33
Rice straw	1.5 % H_2SO_4,130 °C, 30 min, S: L = 1:10	Xylose, 17.2; glucose, 4.3; arabinose, 3.3	Acetate, 1.43; HMF, 0.15; furfural, 0.25
Corn stover	2.13 % H_2SO_4,180 min, 121 °C, S: L = 1:10	Xylose, 9.09; glucose, 2.13; arabinose, 1.01	Acetic acid, 1.48; furans, 0.56; phenolics, 0.08
Spruce	Sulfur dioxide (1 kg), 40 kg wood chips, 203 °C, 5 min + enzymatic hydrolysis	Glucose, 82.4 ± 2.9; xylose, 9.2 ± 0.1; galactose, 3.8 ± 0.3; mannose, 26.4 ± 0.7; arabinose, 2.9 ± 0.2	Phenolics, 0.44 ± 0.05; furfural, 1.0 ± 0.1; HMF,3.3 ± 0.2; acetic acid, 5.0 ± 0.4; levulinic acid, 0.2 ± 0.1; formic acid, 0.7 ± 0.1
Poplar	AFEX treatment + acid hydrolysis	Glucose, 2.0; xylose, 11.36; arabinose, 0.49	2-Furoic acid, 0.3 µg/g; 3,4-HBA, 2.5; salicylic acid, 56; syringaldehyde, 6.0; ferulic acid, 4.7
Soft wood	2 % HCl + 0.5 % $FeCl_2$(v/v), 170 °C, 30 min	Monomeric sugars, 37.5 g L^{-1}	Acetic acid, 5.3; furfural, 2.2

Table 1.9 Examples of acid hydrolysis, sugars produced, and inhibitory compounds produced.[35]

Furfurals are thought to lower the permeability of the yeast cell membrane, and damage DNA with the effect of slowing growth and reducing ethanol productivity.[35, 279] HMF and furfural are reported to be the most toxic of the inhibitors to most yeasts, especially in combination.[137] These inhibitors disrupt cell growth by reducing the activity of various enzymes involved in respiration, damaging DNA and in so doing disrupt DNA and RNA synthesis.[280, 137] During the lag phase *S. cerevisiae* converts furfural into furoic acid.[279]

The mechanism of inhibition of all the phenolic lignin decomposition products is uncertain, however it is thought that they become incorporated into the cell membrane,

leading to its losing its selective permeability and then swelling.[270, 272, 280] Diffusion of the acids through the cell membrane leads to a lower intracellular pH by disrupting the action of the ATPase proton pump which leads to enzyme denaturing.[272, 281] Acetic acid in particular triggers the cell death mechanism (apoptosis).[280] Many of these inhibitors lead to oxidative stress through formation of reactive oxygen species.[280]

In order to reduce these effects, lignocellulose hydrolysates are sometimes detoxified (i.e. the concentration of the inhibitors is reduced) prior to fermentation. One of the most commonly cited methods is overliming - the addition of calcium hydroxide to around pH 10 and removal of the sediment that forms. After acidification to the fermentation pH (typically around pH 5), more sediment is removed.[137, 189, 282] Filtration over activated charcoal is a common method to remove impurities of all kinds, and has been applied to the detoxification of lignocellulose hydrolysates (Table 1.10).[137] An alternative method is by using vacuum membrane distillation,[283] and the hydrolysate concentrated by evaporation of water.[189]

Detoxification may be biological, using enzymes such as laccase that degrades aromatic compounds, or by adding microbes such as *Trichoderma reesei* (a soft-rot fungus), but this is slow.[35, 271] Another option is to genetically engineer the fermenting yeasts to be better able to handle these compounds, such as inserting a gene from *Myceliophthora thermophyla* into *S. cerevisiae* to increase the production of laccase.[35]

Inhibitor	Before (g L^{-1})	After (g L^{-1})
Furfural	0.12	0.06
5-HMF	0.42	0.32
Acetic acid	1.40	0.04
Lignin (soluble)	5.19	1.83

Table 1.10 Example of inhibitor content before and after detoxification by overliming then filtration over active charcoal.[132]

One problem with detoxification is that the sugar content can be reduced substantially.[284] The sugar content of a hemicellulose hydrolysate produced by Tao *et al.* lost 35% of its sugar content after removal of volatile compounds and overliming[189] whereas the wheat straw hydrolysates prepared by Chen *et al.* lost 26%.[137] Adding stages to the depolymerisation also increases the material inputs and capital costs, as well as being energy intensive.[285] It is therefore desirable to use a yeast strain with a high inhibitor tolerance that would nullify the need for such steps.

Inhibitor tolerance varies between species. Some non-oleaginous yeasts strains (e.g. *Pichia stipitis* NRRL Y-7124 and *S. cerevisiae* NRRL Y-12632) are more tolerant than the wild strains to furfural and HMF. Tolerant strains appear to use NADH[286] to reduce the aldehyde group of HMF and furfural[272] to presumably less toxic alcohols (i.e. 2,5-bis-hydroxymethylfuran).[287] They also deal with oxidative stress and problems in protein folding more vigorously.[280, 288] Inhibitor tolerant yeasts have been found to reduce their rate of nitrogen metabolism in response to inhibitors.[288] Aromatic inhibitors may have their highly oxidised aldehyde and carboxylic acid and alkene groups reduced, such as the reduction of coniferyl aldehyde to coniferyl alcohol by *S. cerevisiae*.[289]

The growth of *Cryptococcus curvatus* is inhibited by 72% by the presence of 1 g L^{-1} furfural.[84] *C. curvatus* is also hypothesised to convert furfural to the less toxic furfuryl alcohol. *C. curvatus* has been described by Chen *et al.* as tolerant of inhibitors, yet its biomass and lipid content reduced by the presence of 1 g L^{-1} furfural and HMF to below 50% of a control without inhibitors.[137]

Huang *et al.* studied a comparatively inhibitor tolerant oleaginous yeast, *Trichosporon fermentans.*[290-292] Although *T. fermentans* can grow in the presence of inhibitors below 5 mM, both growth and lipid accumulation are reduced by over 50% at levels higher than this. *T. fermentans* is most sensitive to furfural and vanillin, and least sensitive to 5-HMF. Variation of growth temperature, inoculum size and initial pH with each of the five aldehyde inhibitors studied suggested that varying these factors partially mitigated the effect on the lipid yield of the individual inhibitors, but the effect was inconsistent. They also concluded that furfural was metabolised, as it was not present at the end of fermentation, suggesting that furfural is converted into furoic acid in the same way that it is by *S. cerevisiae*.[135, 277] Huang *et al.* also studied the effect of the inhibitors on the malic enzyme, which is needed to provide NADPH to microorganisms, and was inhibited by furfural, 4-HBA and syringaldehyde, but not vanillin. NADPH is a requirement for fatty acid synthesis, and (see 1.2.2 above), so this may explain the reduced rate of lipid formation in the presence of inhibitors. The rate of glucose consumption was also decreased, in the presence of aldehydes, but in the presence of vanillin and syringaldehyde, the consumption of xylose was relatively higher after 7 days.

The inhibitors did not alter the fatty acid composition of the lipid extracted. As almost all this work has been undertaken on the individual inhibitors, it is difficult to extrapolate the optimal growth conditions in the presence of multiple inhibitors. However, despite the presence of inhibitors, lipid yield was in the region of 12 g L^{-1} after 10 days.[290-292]

1.3.3.3 Increasing resistance to contamination

Contamination of microbial cultures by more robust, less productive microorganisms is potentially catastrophic on an industrial scale, as the yeasts grow less effectively, product purity is reduced and so production must be halted to allow sterilisation and cleaning.[49, 293-296] Such problems often become apparent at the pilot scale, such as NREL's microalgae for biodiesel project (Aquatic Species Program) that was halted due to contamination,[49] and a pilot scale production of ethanol from corn waste using *S. cerevisiae*.[295] Using completely aseptic conditions requires careful handling and containment of the culture media, which increases the capital and running costs of the process.[191, 297] Additionally, the continuous processes that are desirable for the cost efficiency arising from their higher productivity per unit volume, reduced down-time for cleaning and filling the vessels as well as reduced labour costs, are particularly susceptible to bacterial contamination.[238, 295] As a consequence, most industrial scale yeasts fermentations are not run under perfectly aseptic conditions, and contamination is frequent.[294, 295] Resistance to contamination is also desirable as it would allow the use of unsterilized wastewater in the fermentation media, which provides the yeasts with a low-cost, freshwater-saving source of nutrients and water.[298]

The most frequent contaminants in ethanol fermentations are bacteria of the *Lactobacillus* genus,[296, 299] which contaminate the product with lactic acid, and acetic acid producing bacteria.[294, 300] These bacteria can be particularly difficult to control if they catabolise sugars that the yeasts does not, such as arabinose, compounding the problem of limited sugar catabolism.[295] Other wild yeasts may also contaminate the fermentation broth, in particular *Dekkera bruxellensis*.[296] The species which contaminate yeast cultures grown aerobically for oil rather than anaerobically for ethanol, are unclear though contamination was an issue for Lindner's early experiments.[88]

A number of solutions have been investigated to combat contamination including improved process conditions, selection of more robust strains adapted for conditions few other species can tolerate, or adding compounds to the media which inhibit the growth of other microorganisms. The fermentation media is kept free from contamination by heating the inputs and using steam or an antimicrobial (such as ethanol) on the lines and vessels. This process can be improved by pasteurizing the media more aggressively, monitoring the process for very low levels of contamination and isolating the source of contamination to allow for more thorough cleaning and sterilisation of the affected areas.[295, 301]

Selection of a thermophilic strain allows fermentations to be run at temperatures of around 40-70 °C - warm enough to be unsuitable for non-thermophilic species. However, very few yeasts can grow at this temperature so this is a more suitable strategy for bacteria.[302, 303] Similarly, acidophiles capable of growing at pH 4 and below have been used in the laboratory-scale production of the artificial sweetener xylitol by *Candida tropicalis* at pH 2.5,[304] and in the production of ethanol from lignocellulose by *S. cerevisiae* at pH 4.[305] Robust species tolerant to comparatively high temperature, high salt content and low pH were assessed by Kodama *et al.*[303] *Issatchenkia orientalis* was particularly adept at both growing and producing ethanol (2.0 ±0.1% w/w) on several sugars at low pH (2.5) and high salt content (2.5%) at 30 °C, or at slightly less osmotically pressurised conditions (pH 4.0 and salt content 1.0%) at a higher temperature (37 °C). Microbes found in local soil were unable to grow in either of these conditions. However high salinity media may be corrosive to even stainless steel fermentation tanks at concentrations above 0.1%.[196]

The addition of expensive antibiotics and antimicrobials is effective.[157, 295, 299] The far more cost effective method of adding sodium chloride (25 g L^{-1}) and ethanol (12.5 g L^{-1}) was proposed by Albers *et al.* and demonstrated in an industrial scale lignocellulose-to-ethanol plant.[294] Although this did reduce the bacterial viability to near-zero levels after thirty hours and increase ethanol yield, such a technique would be ineffective in a yeast strain intolerant to ethanol or high osmotic pressure and corrosion is still an issue. Similarly, the concentration of inhibitors found in lignocellulose hydrolysate (1.3.3.2 above) is sometimes deliberately raised to levels an inhibitor tolerant yeast can withstand, but bacteria cannot.[210, 295]

As cost of production process for a palm oil substitute needs to be kept so low, additives (especially ones that corrode fermenters) are unacceptable, and high inhibitor concentrations decrease lipid yield, and so a yeast that can tolerate contamination is desirable. In oleaginous species, growth and lipid production are not always affected by contamination, at least at lab scale, such as the case of *R. glutinis* grown on non-sterilised and sterilised wastewater, however in this example growth was poor under both conditions.[298]

1.3.4 Examples of growth of oleaginous fungi and yeasts on lignocellulose hydrolysates

In the last seven years, over twenty studies of the growth of oleaginous fungi on lignocellulose hydrolysates have been published (Table 1.11.)

Strain	X ($g\ L^{-1}$)	LC (%)	LY ($g\ L^{-1}$)	T (°C)	t (h)	pH	Feedstock	N source	Mode	Hydrolysis	Detoxification	Ref.
							Non-yeast fungi					
A. oryzae A-4	4.3	18.6	0.8	30.0	144	5.5	Microcrystalline cellulose	YE+AmS	B	None	NA	[104]
	6.7	25.4	1.7	30.0	144	6.0	Wheat straw + bran	AmS	SS	None	NA	[104]
Microsphaeropsis sp.	7.5	34.7	2.6	30.0	144	6.0	Wheat straw	YE	B	Steam explosion	None	[250]
	78.6	10.7	8.4	30.0	216	6.0	Wheat straw	YE	SS	Steam explosion then enzymatic	None	[108]
M. isabellina ATHUM 2935	5.6	64.3	3.6	28	240	6-6.4	Rice hulls	None	B	Dilute acid	None	[306]
M. isabellina ATCC 42613	12.84	24.8	3.18	25	88	6	Corn Stover	YE	B	Dilute acid then enzymatic	None	[307]
	12.55	35.1	4.4	25	118	6	Switchgrass	YE	B	Dilute acid then enzymatic	None	[307]
	12.28	30.2	3.71	25	118	6	Miscanthus	YE	B	Dilute acid then enzymatic	None	[307]
	13.75	22.0	3.02	25	136	6	Arundo donax	YE	B	Dilute acid then enzymatic	None	[307]
							Yeasts					
C. curvatus ATCC 20509	15.6	27.1	4.2	28	144	5.5	Wheat straw	YE	B	Dilute acid	Overliming	[137]
	27.7	43.6	12.0	30	71	5.5	Corn stover	None	B	Alkali PT then enzymatic	None	[157]
	NS	NS	11.9	30	71	5.5	Corn stover	YE+AmS	SS	Alkali PT, SSF	None	[157]

Strain	X ($g\ L^{-1}$)	LC (%)	LY ($g\ L^{-1}$)	T (°C)	t (h)	pH	Feedstock	N source	Mode	Hydrolysis	Detoxification	Ref.
C. curvatus ATCC 20509	47.3	31.9	15.1	30	71	5.5	Corn stover	None	SS	Alkali PT, SSF	None	[157]
	6.9	31.9	2.2	30.0	120	7-10	Wood	YE	B	Pyrolysis	Water wash	[308]
C. humicola UCDFST101004	35.93	43.06	15.47	30	168	5.5	Corn stover	None	B	Ammonia explosion	None	[125]
C. humicola UCDFST12717	37.05	34.87	12.92	30	168	5.5	Corn stover	None	B	Ammonia explosion	None	[125]
C. laurentii UCDFST12803	16	28	5	30	168	5.5	Corn stover	None	B	Ammonia explosion	None	[125]
G. fermentans CICC 136	28.6	40.1	11.5	25.0	192	6.5	Rice straw	YE + peptone	B	Dilute acid	Overliming + adsorption	[282]
L. starkeyi ATCC 12659	12.7	29.1	3.7	28	144	5.5	Wheat straw	YE	B	Dilute acid	Overliming	[137]
R. glutinis ATCC 204091	11.8	20.7	2.4	28	144	5.5	Wheat straw	YE	B	Dilute acid	Overliming	[137]
R. glutinis CBS 20	11.8	11.9	1.4	30	96	6	Wheat straw	AmS + YE	B	Concentrated acid	None	[163]
R. glutinis	11.8	7.9	0.93	30	96	6	Miscanthus straw	AmS + YE	B	Concentrated acid	None	[163]
	18.09	34.16	6.18	28	96	6	Populus leaves	YE + peptone	B	Dilute acid	Overliming	[189]

Strain	X ($g\ L^{-1}$)	LC (%)	LY ($g\ L^{-1}$)	T (°C)	t (h)	pH	Feedstock	N source	Mode	Hydrolysis	Detoxification	Ref.
R. graminis DBVPG 7021	48	34	16	30	72	6	Corn stover	Corn steep + AmS	B	Dilute acid then enzymatic	none	127
R. minuta 62 NCYC	4.7	4.4	0.03	28	168	5.5	Miscanthus	None	B	Alkali PT then enzymatic	none	128
R. mucilaginosa	13.84	42.63	5.9	25	72	6	Wheat straw	YE + AmS	B	Dilute acid	None	309
	16.21	38.25	6.2	25	72	6	Rice bran	YE + AmS	B	Dilute acid	None	309
	15.3	49.02	7.5	25	72	6	Grass	YE + AmS	B	Dilute acid	None	309
	15.07	45.12	6.8	25	72	6	Leaves	YE + AmS	B	Dilute acid	None	309
R. toruloides ATCC 10788	9.9	24.6	2.4	28	144	5.5	Wheat straw	YE	B	Dilute acid	Overliming	137
T. cutaneum CX1	10.2	30.5	3.11	25	120	5-5.5	Corn stover	AmS	B	Dilute acid	Biological	190
	NS	NS	3.23	30	78	5	Corn stover	AmS	B SSF	Dilute acid then enzymatic	Biological	310
T. cutaneum ACCC 20271	38.4	32.0	12.3	30	120	5	Corn cobs	AmS + corn steep liquor	B	Xylitol extraction then enzymatic	Washing	133
T. cutaneum CH002	22.1	36.0	8.0	28	192	5.5	Corn cobs	None	B	Dilute acid	Overliming and adsorption	311
T. coremiiforme CH005	20.4	37.8	7.7	28.0	192	7.0	Corn cobs	None	B	Dilute acid	Overliming	132
T. dermatis CH007	24.4	40.1	9.8	28	168	7	Corn cobs	None	B	organic solvent PT enzymatic	None	135

Strain	X ($g\ L^{-1}$)	LC (%)	LY ($g\ L^{-1}$)	T (°C)	t (h)	pH	Feedstock	N source	Mode	Hydrolysis	Detoxification	Ref.
T. guehoae UCDFST 60-59	10.4	25.9	2.7	30	120	5.5	Corn stover	None	B	Ammonia explosion	None	[125]
Y. lipolytica ATCC 20460	7.2	4.4	0.3	28	144	5.5	Wheat straw	YE	B	Dilute acid	Overliming	[137]
Y. lipolytica Po1g	11.42	58.5	6.68	26	120	6.5	Sugarcane bagasse	Peptone + YE	B	Dilute acid	Overliming	[138]

Table 1.11. Oleaginous yeast species (and strains where given in reference) cultivated on lignocellulose hydrolysates and their conditions including (from left to right) dry cell mass (X), lipid content of dry cell mass in % wt/wt (LC) , lipid yield (LY, temperature (T), time (t), pH at the start or (where known) during culture, the lignocellulosic feedstock used, the form of nitrogen added including yeasts extract (YE) and ammonium sulfate (AmS), the mode of operation, whereby B = batch, SS = solid state, SSF = simultaneous saccharification and fermentation, the method of lignocellulose hydrolysis including the pretreatment step (PT) where applicable, the method of detoxification and the reference. NS = not stated, NA = not applicable

Three moulds have been demonstrated to convert wheat straw effectively to oil by secreting cellulases and xylanses in solid or semi-solid state consolidated bioprocess (Table 1.11, entries 1,4,5).[104, 108, 250, 312]

Trichosporon coremiiforme was selected for its ability to assimilate xylose. It grew impressively on dilute-acid hydrolysed corncob, consisting of 37.9 $g\ L^{-1}$ xylose, 2.9 $g\ L^{-1}$ glucose and 4.9 $g\ L^{-1}$ arabinose (Table 1.11, entry 35). No additional nitrogen sources or nutrients were added, aside from the yeasts extract and peptone in the inoculum, which was added at 10% vol. However, the hydrolysate was first de-toxified by overliming and filtration over activated charcoal. This reduced the furfural content to 0.06 $g\ L^{-1}$ and the HMF to 0.32 $g\ L^{-1}$. Nevertheless, the cell mass (10.4 $g\ L^{-1}$) and lipid content (24.2%) were lower in the non-detoxified medium that contained a total of 1.53 $g\ L^{-1}$ furfurals.[132] In the same genus, *Trichosporan dermatis*, (entry 36) has also been found to grow on corncob hydrolysate to yield 9.9 $g\ L^{-1}$ lipid. It benefited from the pretreatment being with only organic solvents, and so the authors declared that no inhibitors were present, though any experimental evidence was not presented.[135]

Trichosporon cutaneum has been shown to grow on both hydrolysed corn cob[133, 311] (entries 33 and 34) and corn stover[190, 310] (entries 31 and 32). The corncob feedstock (entry 33) had had xylitol extracted from it, so was already physically and chemically broken-down prior to enzymatic hydrolysis and contained only 6-carbon sugars. *T. cutaneum* grew on this feedstock, whereas *R .glutinis* CGMCC 2.703, *R. glutinis* CGMCC 2.704, *L. starkeyi* DSM 70295 and *R. toruloides* CGMCC 2.1609 did not, indicating that these yeasts were less tolerant of the presence of 1.0 g L^{-1} of acetic acid and 0.15 g L^{-1} of phenolic compounds. A strain of *T. cutaneum* with improved inhibitor tolerance was created by irradiation with UV light. This mutant, *T. cutaneum* CX1, yielded 3.11 g L^{-1} lipid on corn stover hydrolysed by dilute acid hydrolysis and biologically detoxified with *Amorphotheca resinae* ZN1 spores. Despite the use of a mutant strain, this yeast only grew in the hydrolysates that had all the furfurals removed, which took seven days.[190] Nevertheless, this procedure was scaled up to 50 L and modified to a SSF process. This increased the lipid yield only slightly (3.23 g L^{-1}), but it did reach this level in two fewer days demonstrating the efficiency of SSF.[310]

Trichosporan fermentans (entry 18) [282] was grown on rice straw hydrolysed by dilute acid hydrolysis, and detoxified by overliming and filtration over the resin Amberlite XAD-4, as well as concentration of the medium by vacuum distillation. This detoxification process improved the performance of the yeasts, increasing the dry cell mass from 11.4 g L^{-1} to 28.6 g L^{-1}, the lipid content from 14.6% to 40.1% and the lipid yield from 1.7 g L^{-1} to 11.5 g L^{-1}. Huang *et al.* observed that lipid was slower to accumulate than when grown on pure glucose,[142] and attributed this tardiness to the presence of inhibitors. The stored lipids appear to begin being used by the yeasts beyond day eight as the lipid content decreased, demonstrating that there is a need to select an optimum fermentation time.

Yarrowia lipolytica, cultured on dilute acid hydrolysed sugarcane bagasse, accumulated 6.68 g L^{-1} lipid despite its limitation in terms of sugar utilisation (entry 38). In order to achieve this result, the hydrolysate had to be detoxified and supplemented with 5 g L^{-1} yeast extract and 5 g L^{-1} peptone. Presumably, these nitrogen sources, especially at this concentration, would be too costly for an industrial fermentation if lipids were the only product. Interestingly, the yeast grew better on the hydrolysate than a synthetic glucose based medium, both of which contained 20 g L^{-1} sugar; the authors reasoned that this strain of *Y. lipolytica* appeared to be more efficient at transporting xylose into the cell than glucose.[138]

Boundy-Mills *et al.*[125] screened 39 yeasts on model corn stover hydrolysate (that contained no inhibitors but did contain xylose as well as glucose). For the nine yeasts that grew well on this medium, the authors demonstrated that the yeasts consumed both

glucose and xylose faster after pre-culturing on xylose, presumably, as the yeasts have already activated the metabolic pathways for catabolising xylose so the necessary enzymes are already synthesised. This appears to correlate to a greater cell density and lipid yield, although no quantitative analysis was undertaken. Three *Cryptococcus* strains were selected for scale up to 100 mL on actual corn stover hydrolysate, pretreated with ammonia and hydrolysed enzymatically. These included two strains of *C. humicola* one of which gave the second highest lipid yield of oleaginous yeast grown on lignocellulose (lipid yield: 15.47 g L^{-1} and 12.92 g L^{-1}) and a newly identified oleaginous strain; *C. laurentii*, which gave less impressive lipid yields (4.5 g L^{-1}).

Five yeasts were screened in dilute acid hydrolysed wheat straw by Chen *et al.*[137] Strangely, three of these yeasts performed better in terms of lipid yield in the non-detoxified hydrolysate, including the best performer - *C. curvatus*. This yeast was studied further and found to be using its capability to metabolise acetic acid[159] (initial concentration = 4.0 g L^{-1}) and remove furfural (initial concentration 0.4 g L^{-1}), both of which dropped to a near zero concentration after 24 hours. The overall conversion of straw to lipid was 4.7%.

C. curvatus has also produced oil from corn stover hydrolysate that was pretreated with alkali to remove lignin then enzymatically hydrolysed. A direct comparison between SHF and SFF was carried out by Zhao *et al.*[157] SSF took place in semi-solid state conditions, with corn stover (10% w/w) in a phosphate buffer. As the lipid yield without additional nitrogen was unsatisfactory (6.2 g L^{-1}), ammonium sulphate was added to improve the yield, which was effective (11.9 g L^{-1}) until too much was added which reduced the lipid content (to 11.1 g L^{-1}).. Hydrolysis in absence of additional nitrogen or β-glucosidase enzymes was the most efficient in terms of the lipid produced per unit mass of corn stover. This is because *C. curvatus* has been shown to be able to catabolise some oligomeric polysaccharides,[251] making these conditions more like consolidated bioprocessing than SSF. *C. curvatus* has also been shown to grow and produce lipid in non-sterilised corn stover under simultaneous saccharification and fermentation conditions only slightly less well than sterilised conditions (11.9 versus 12.5 g L^{-1}). The absence of other nutrients and large inoculum size was thought to prevent contamination.[313]

Rhodotorula glutinis was found to grow and produce lipid poorly on non-detoxified wheat straw (and worse on miscanthus straw) hydrolysate by Graeff-Hönninger *et al.*, reaching only 1.8 g L^{-1} lipid.[163] The yeast's sensitivity to inhibitors and inability to utilise sucrose were highlighted as reasons for this low yield. *Rhodotorula glutinis* was grown on hemicellulose-rich leaves hydrolysed by dilute acid, yielding 6.18 g L^{-1} lipid, demonstrating that it is possible to achieve reasonable yields of lipid without the addition of enzymes.

This requires selecting a feedstock with a high hemicellulose content – in the case of the popular leaves used in this study, 80-85% of their dry weight.[189]

Rhodotorula graminis (entry 24) gave the highest lipid yield to date from an oleaginous yeast grown on lignocellulose at 16 g L^{-1}. This yeast was particularly effective as it catabolised a range of sugars and was to some extent tolerant of inhibitors, as demonstrated by their concentration reducing to near zero levels in the first 48 hours of fermentation. However, inhibitors in isolation showed that the growth was slowed by 55% and up to 100% by 5-HMF and furfural respectively, suggesting that the optimum fermentation time may have been longer than the 72 hrs allowed. Lipid content was also lowered by the addition of inhibitors. Corn stover hydrolysate was also used under fed-batch conditions, but instead of feeding with hydrolysate, was fed with 400 g L^{-1} glucose solution, resulting in a lipid yield of 50 g L^{-1}.[127]

1.4 Metschnikowia pulcherrima

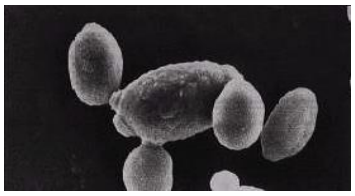
One of the oleaginous yeasts that has characteristics in line with what is required for growth on lignocellulose hydrolysates that has not been studied for this purpose is *Metschnikowia pulcherrima*. Lindner noted the presence of fat droplets in what he originally named *Torulopsis pulcherrima* in 1901.[314] The yeasts was re-designated to the genus *Metschnikowia* by Pitt and Miller in 1968[86] and its taxonomic classification makes it a member of the phylum Ascomycota, the class *Saccharomycetes*, the order *Saccharomycetales* and the family *Metschnikowiaceae*. This means it is far more closely related to *L.lipofer*, *S. cerevisiae* and *Y. lipolytica* than the basidiomycetes such as the *Rhotodorula*, *Trichosporon*, *Rhodosporidium*, or *Cryptococcus* species.[315] Generally basidiomycetes are more tolerant of different sugar substrates and less likely to need additional vitamins than ascomycetes,[84] but this does mean that the biochemistry known of *S. cerevisiae* and *Y. lipolytica* is more likely to be applicable to *M. pulcherrima*. *M. pulcherrima* strains are common and found worldwide, typically on the surface of fruit (notably grapes).[316]

Despite Lindner's observation, the yeast has been primarily of interest in wine making, and for its red pigment- pulcherriminic acid.[314, 317] Pulcherriminic acid is an antimicrobial compound that chelates iron from its surroundings, depriving other organisms of this vital nutrient, and meaning *M. pulcherrima* has been suggested for use as a biotic post-harvest pest control.[318, 319] Importantly, *M. pulcherrima* is acidophilic[316] and so is capable of thriving in media of pH 3 – an environment too harsh for almost all bacteria to survive. Some strains have been shown to kill off *S. cerevisiae* as the pH is lowered.[316] *M. pulcherrima* therefore has the exciting potential to outcompete contaminating

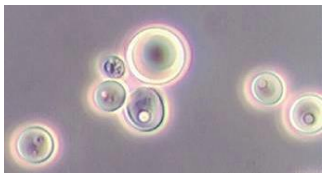
microorganisms, enabling less sterile control on fermentations (see section 1.3.3.3). Although generally non-pathogenic, there are three reported incidences of *M. pulcherrima* infections in a extremely immunocompromised person, a lung condition and a premature infant.[320-322]

Recently, *M. pulcherrima* grown in glycerol (140 hours, 28 °C, C/N = 66, pH 6) had been described as non-oleaginous by Papanikolau *et al.* finding only 0.6 % lipid.[95] Taccari *et.al.* confirmed that *M. pulcherrima* could metabolise glycerol and reported that the maximum amount of biomass that could be derived from glycerol was 22 g L^{-1} at 27 °C and 60 g L^{-1} glycerol loading, however the lipid content was not determined.[255] Several pre-1970 papers mention the presence of a "single large oil droplet"[86] within the cells, but this was not quantified or chemically analysed. However, in 2009, Pan *et al.* established that *M. pulcherrima* was capable of producing lipid at 30% by weight of dry mass, and that it could do this while metabolising both xylose and glycerol.[143]

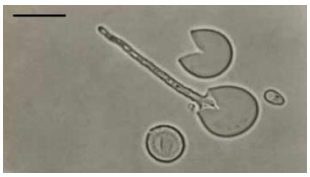
The life cycle of *M. pulcherrima* like other yeasts (see 1.2 above) involves exponential growth via asexual budding after inoculation to form vegetative cells (Figure 1.8a). *M. pulcherrima* can reproduce by forming spores (fig 6c), and in process leading up to spore formation, it accumulates lipid in droplets and the cell walls thicken (1.8b).[86] Therefore, it seems likely that *M. pulcherrima,* could produce lipids from complex waste resources while potentially being able to ward off invasive species.



a) vegetative *M. pulcherrima*



b) oil-rich *M. pulcherrima* cells



c) needle-shaped spore emerging from a *M. pulcherrima* cell

Figure 1.8 Micrographs of *M. pulcherrima* cell types at various stages of its life cycle

1.5 Aim and objectives

The aim of this work is to investigate and further develop *M. pulcherrima* as a suitable organism for the industrial production of a microbial palm oil substitute. The working hypothesis is that *M. pulcherrima* is a suitable organism for use in industrial biotechnology, particularly for lignocellulose conversion to lipid.

From the literature reviewed above it appears that for an industrially relevant oil-producing organism cost minimisation is crucial, as well as environmental and social sustainability. This can be achieved through selection of process conditions and a yeast that is suitable for generating lipid in these conditions.

The process should:

- Use a second generation feedstock with near zero land use change that can be sustainably harvested and converted into sugars cheaply with minimal energy and substrate requirements
- Use consolidated bioprocessing, which is potentially a cheaper and more productive route
- To eliminate extraction costs and environmental harms of solvents, create a product that uses all the components of the yeast without extracting the oil
- Avoid or minimise the use of additional enzymes, a large factor in environment al burden and cost.

Consequently, the yeast should:

- Thrive in non-sterile conditions
- Not be genetically modified
- Utilise a wide range of sugars, in particular those found in lignocellulose
- Be able to tolerate inhibitors at the levels produced in the hydrolysis process
- As an additional advantage, produce valuable by-products
- produce cellulases, xylanases or β-glucosidases.

In order to be comparable to the median values of oleaginous yeast cultivated on lignocellulose:

- lipid content should be over 20%, ideally 40%; lipid yield should be over 2 g L^{-1}; lipid productivity should be over 0.7 g L^{-1} d^{-1}; dry cell mass should be over 13 g L^{-1}.

To achieve this aim the work has three key objectives:

1. The first objective is to determine whether *M. pulcherrima* is suitable for the production of lipids from waste resources. This includes the ability to consume multiple sugars, whether it can be made to grow oleaginously and whether the ability to produce antimicrobials allows the yeast be cultured under non-sterile conditions.
2. On establishing the suitability of *M. pulcherrima* for the production of lipids, the ability to be cultured on lignocellulosic hydrolysate will be investigated. Focus will be given to non-enzymatic depolymerisation techniques.
3. As one of the main uses of palm oil in the UK is in care product emulsions, the ability of the products, as well as the whole cells, will be investigated for the ability to form stable emulsions.

2 Methods

2.1 Methods common to multiple chapters

2.1.1 Materials and organism

All chemicals were purchased from Sigma Aldrich unless otherwise stated and used without purification. Spring wheat straw was obtained from a farm local to Bath, UK and stored at 18 °C in a sealed plastic container in the dark. Samples of straw were reduced in size by cutting with scissors to >1 cm in size pieces, then grinding in a food blender for 5 minutes, the fraction that could pass through a 1.2 mm sieve was retained. Lignin was alkali linin derived from the SHOP process. Rapeseed oil was purchased from a local supermarket. *M. pulcherrima* was obtained from the National Yeast Culture Collection (Norfolk, UK) and stored on sterile YPD agar plates at 4 °C. *M. pulcherrima* was re-plated every two months to ensure the cultures remained viable and uncontaminated.

2.1.2 Batch liquid hot water/acid hydrolysis of wheat straw

Based on the method of Yu *et al,*[137] pelleted wheat straw (50 g) was combined with DI water (800 ml) or with 0.1% mM sulphuric acid in 1L Duran bottles, loosely sealed and autoclaved at 120 °C for 1 hour. After cooling, solids were removed by centrifugation, and washed with 40 ml portions DI water until the total volume of hydrolysate removed equalled 800 ml.

2.1.1 Yeast cultivation

Unless otherwise stated, the control medium was yeast minimal medium (YMM), optimised previously,[323] (Table 2.1). The medium was prepared in deionised water in a Duran bottle (without adding calcium chloride), the pH was then adjusted to 5 using concentrated hydrochloric acid, then the calcium chloride added, then the Duran bottle was autoclaved at 121 °C, 20 min, unless otherwise stated.

Component	Concentration (g L^{-1})
H_2KPO_4	7.00
Na_2HPO_4	2.50
$MgSO_4.7H_2O$	0.188
$MgCl_2.6H_2O$	1.08
$ZnSO_4.7H_2O$	0.0200
$(NH_4)_2SO_4$	0.0625
NH_4Cl	0.354
Glucose/glycerol	30.0
Yeast extract	1.00
$CaCl_2.2H_20$	0.150

Table 2.1. Optimised yeast minimal medium (YMM) for *M. pulcherrima.*[329]

Cultivation was carried out under sterile conditions unless otherwise stated. Media and non-irradiated fermentation vessels were autoclaved at 121 °C for 20 min prior to use and handled using aseptic techniques in a laminar flow hood. Innocula were prepared in sterilised YPD (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose) from a single colony of *M. pulcherrima* , incubated at 25 C for 24 hours, then diluted to an OD_{600nm} of 0.6 with sterile YPD. Where YMS (30 g L^{-1} yeast extract, 5 g L^{-1} mannitol, 5 g L^{-1} sorbose) was used, the same method was applied. Sterile vessels were charged with minimal medium to the volume specified in a 1:5 media to air ratio. Cultivation was carried out in the dark in incubators set to 25 °C ± 1 °C shaken at 180 rpm unless otherwise specified.

Optical density at 600 nm was measured after diluting a 10-50 µl sample in DI water by at least a factor of 10, using a UV-vis spectrophotometer (Perkin-Elmer), blanked to DI water for control medium or to the hydrolysate prior to inoculation at the same dilution.

2.1.2 Analytical methods

Glucose, xylose, cellobiose, arabinose, acetic acid, formic acid, levulinic acid and arabitol content were determined by Shimadzu 10AVP HPLC system (Shimadzu corp., Japan) fitted with a pump (LC-10AD), an auto injector (SIL-10AD), a system controller (SCL-10A). Filtered (0.22 µm, Millipore, UK) hydrolysate samples (10 µl) were injected without dilution onto a 300 x 7.8 mm Aminex HPX-87H column (BioRad, CA, USA) at 65 °C fitted with RID-10A detector. Isocratic elution took place was over a 25 min period at 0.6 mL/min using 0.2 µm-filtered and degassed 5 mM sulfuric acid. 2-phenylethanol content was determined using an identical HPLC system with a UV detector, filtered samples diluted by a factor of 20 and 10 uL injected onto a Dionex column eluted at 40:60 water:acetonitrile at 0.4 mL min^{-1}.

CHN elemental analysis was carried out in duplicate at London Metropolitan University.

Cell counts were by flow cytometry (Guava EasyCyte) or with a microscope and haemocytometer.

2.1.3 Microplate fermentation

96-well plates were charged with 200 µl 6 replicates of medium, in non-sterile conditions, sterilised by UV light in laminar flowhood for 1 hour, then inoculated with 5 µl YPD inoculum. The lid was sealed with parafilm, an OD reading taken, then the plate was incubated at the specified temperature, and shaken at 180 rpm (except for the fermentation carried out at 22 °C). OD was measured once or twice per day at 600 nm using a microplate reader (either by Versamax, Molecular devices or a Modulus II by Turner BioSystems).

The OD was analysed one well at a time. Firstly the change in optical density (ΔOD) after time (t) was found, and then normalised to the average ΔOD for the control medium $\Delta OD_{(control)}$ to give $norm\Delta OD_{t=x}$

Equation 2.1) $\Delta OD = OD_{t=x} - OD_{t=0}$ equation 2.2) $norm\Delta OD = \frac{\Delta OD}{\Delta OD_{(control)}}$

2.1.4 Cell dry mass

The mass of the dried cells per unit volume of growth medium is defined as the dry cell mass (X, g L^{-1}). The cell dry mass was found by centrifuging the fermentation broth at

6000 rpm, decanting the supernatant, freeze or oven drying (40 °C) the pellet for 24-48 hours then weighing the yeast biomass. Where optical density (OD) used to estimate cell dry mass, a range of optical densities and a calibration curve calculated (

Figure **2.1**). This was only used in glucose and glycerol media, as the lignocellulose hydrolysates absorbed light at a wide range of frequencies.

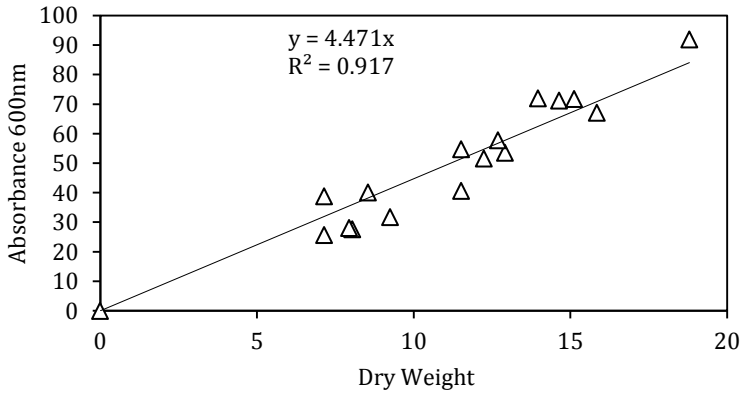


Figure 2.1. Optical density to cell dry mass calibration

2.1.5 Lipid content determination

The standard 'Bligh and Dyer' method was used to extract oils.[324] Briefly: cells were washed three times in DI water then freeze-dried. Yeast (0.1 g) was stirred over 1-7 days in a 2:1 chloroform:methanol solvent mixture or heated in 2:1 chloroform:methanol (5 ml) in a microwave reactor for 15 min at 110 °C. The biomass was filtered, the solvent removed under vacuum. The extracted oil was weighed, and the process repeated until there was no change in the mass of oil.

In chapters 3 and 4, this method was modified based on the procedure of Pan *et al*[143]*.* in which dried yeast biomass of known mass (0.02-0.1 g) was heated at 50 °C in 4M HCL for 60 min, cooled, 10 ml 1:1 chloroform:methanol were added and the mixture was stirred overnight. The chloroform layer was carefully removed by pipette into tared vials, the solvent removed and the vials re-weighed.

This value is called the lipid content (LC, %), and the amount of lipid present in the medium is the lipid yield (LY, g L^{-1}). Lipid productivity is the amount of lipid produced over time (g L^{-1} d^{-1}), a crucial measure for determining the profitability of a potential industrial production process. A nile red and boron-dipyrromethene dye method to stain the cells was also attempted, but calibration was impossible due to other compound strongly absorbing at the test frequencies for both dyes.[129]

2.1.6 FAME profile determination

Oil was transesterified using methanol and concentrated sulphuric acid, washed with water and dried down according to a standard literature procedure.[325] The resulting esters were quantified by GC-MS with the resulting FAME component compared to known FAME standards. GC-MS analysis was carried out using an Agilent 7890A Gas Chromatograph equipped with a capillary column (60m × 0.250mm internal diameter) coated with DB-23 ([50%-cyanpropyl]-methylpolysiloxne) stationary phase (0.25 μm film thickness) and a He mobile phase (flow rate: 1.2mL/min) coupled with an Agilent 5975C inert MSD with Triple Axis Detector. FAME samples were initially dissolved in 2 mL of dioxane and 1 μL of this solution was loaded onto the column, pre-heated to 150°C. This temperature was held for 5 mins and then heated to 250°C at a rate of 4°C/min and then held for 2 mins.

2.1.7 Design of experiments

Experiments were designed using the statistical software package MODDE (Umetrics, Sweden). The design was selected based on its ability to give useful data within experimental limitations, and included 3 replicates at the centre point. The experiments were carried out and the responses recorded.

Models for each response were then defined by examining the correlation between all factors (and interactions between all factors, and squares of interactions with themselves) and the responses. Factors that were insignificant, defined by those whose removal led to the increase in the Q2 value, (a measure of the predictive power of the model) were removed..

In order to examine the accuracy of the models in predicting the responses, the values predicted for each of the experiments are compared to the observed values for these scenarios. If the model were perfect, the predicted values and observed values would be identical, so the correlation between these values would be linear and highly significant. The null hypothesis that there is no correlation between the observed and predicted values was analysed by ANOVA. The response surfaces were then calculated.

2.2 Chapter 3 methods

2.2.1 Nutrient rich medium

The initial medium was identical to that used by Chatzifragkou *et al.* as follows.[95]

Component	Concentration (g L^{-1})	Component	Concentration (g L^{-1})
H_2KPO_4	7.00	NH_4Cl	0.354
Na_2HPO_4	2.50	$MnSO_4.H_2O$	0.06
$MgSO_4.7H_2O$	1.5	$FeCl_3$	0.15
$MgCl_2.6H_2O$	1.08	Glycerol	25
$ZnSO_4.7H_2O$	0.0200	Yeast extract	1.00
$(NH_4)_2SO_4$	0.5	$CaCl_2.2H_20$	0.15

Table 2.2. Nutrient-rich medium composition (OMP), used for initial lipid content vs. temperature experiments.

2.2.2 Repeated batch cultivation

OMP media was prepared using 7.5 g of each of glucose, xylose, arabinose and cellobiose and varying the amount of yeast extract, biotin and additional nitrogen (as per Chapter 3, Table 1). The medium used final effluent waste water from a waste water treatment plant instead of DI water and none of the reagents were sterilised prior to use. Each culture was fed every 2 to 3 days up to 24 days (25°C, 180 rpm) and then allowed to rest at 15°C for 12 days without agitation. Other than yeast extract, biotin and the carbon source all nutrients were supplied according to the OMP media, except phosphate, which was supplemented only at 15 days since the starting concentration greatly exceeded the nutritional requirements of the yeast.

2.2.3 Raceway pond cultivation

YMM with a glycerol content of 30g L^{-1} was used to establish cultures in two 500L capacity raceway ponds situated in a climate-controlled glasshouse. The ponds were inoculated with 500ml of a *M. pulcherrima* culture grown for 48 hours at 25 °C with agitation (180 rpm) in YMS medium consisting of 30g L^{-1} yeast extract, 5g L^{-1} mannitol and 5g L^{-1} sorbose.

The pond cultures were agitated using a close-fitting paddle wheel driven at 10 rpm and aerated through two spargers situated at opposite sides of the ponds. Culture temperature, pH and O.D.600nm were measured daily until the onset of stationary phase, then every 4 days together with the addition of lipid fluorescence up to 28 days. With the onset of stationary phase the temperature in the glasshouse was reduced from 25 °C to 20

°C, the aeration was stopped and the paddle wheels were set at the minimum rotating rate (4 rpm).

2.2.4 Oil properties

The energy density was determined using a bomb calorimeter (6 bar O_2) using 0.2880 g oil to heat 2 L water. The temperature plateaued after 7 minutes, increasing by 0.73 °C.

Dynamic viscosity was determined using a Bholin rheomter, flat plate mode at 40 °C.

^{1}H NMR spectroscopic measurements were carried out at 298 K using a Bruker AV500 spectrometer, operating at 300 MHz. ^{1}H spectra were typically acquired using a 30 degree excitation pulse and a repetition time of 4.2 sec. 0.3 Hz line broadening was applied before Fourier transform, and spectra were referenced to the residual $CHCl_3$ peak from the solvent (δ 7.26 ppm). The lipid and sterol content were calculated by comparison of the glyceride protons (δ 4.1ppm) to the α-protons of the sterol alcohol group (δ 3.7-3.9 ppm).

2.2.5 Wheat straw compositional analysis

Analysis of the main components (cellulose, hemicellulose and lignin) within the wheat straw was carried out using a method adapted from Brindha et al.[326] Mechanically pretreated wheat straw (2 g) was boiled in ethanol (50 mL) for 15 minutes, four times. The solid was washed with water and oven dried overnight (60 °C). The remaining dry mass was determined and labelled the A fraction.

The A fraction was then treated with 24 % (w/w) potassium hydroxide for 4 hours at 25 °C before it was filtered and washed thoroughly with DI water. The oven dried solid was weighed and labelled B.

The B fraction was treated with 95-97 % sulphuric acid for 3 hours to hydrolyse the cellulose. The treated sample was then refluxed with 5 % sulphuric acid for a further 2 hours. The sample was filtered, washed and then oven dried (70 °C) overnight. The remaining dry mass was labelled the C fraction. The fractions within the wheat straw could then be determined as follows: non-lignocellulosic material = A, cellulose = B-C; hemicellulose = A-B; lignin = C

2.2.6 Inhibitors

Inhibitor concentrations were selected based on the typical range of values found in lignocellulose hydrolysates,[35, 275] which are typically reported in g L^{-1}, and so in order to compare inhibitors, these were converted into molar concentrations, assuming the mass of each inhibitor was equivalent to that of 5-HMF. Microplates were charged with media contaminated by inhibitors and fermentation was carried out as described above.

2.2.7 Acid hydrolysis of wheat straw

Dilute acid hydrolysis of size-reduced wheat straw (1 g) was carried out using formic, acetic, malonic, levulinic, oxalic and sulphuric acids (20 ml, 200 mM) as well as water in a magnetically stirred (500 rpm) 50 mL bomb reactor (Parr, US), fitted with sand bath pre-heated to 250 °C. The temperature was then set using a thermocouple inserted into a port in the reactor set to 170 °C. The temperature was maintained at 170 °C for 240 min, and sampled at 30 min intervals for the first 2 hours, then at hourly intervals, using the sampling port. The samples were centrifuged at 12000 rpm for 10 minutes, the supernatant decanted, a portion run in HPLC and the remainder frozen. The end of time sample was either filtered or centrifuged to remove residual solid lignocellulose and the supernatant and solid frozen. Samples removed during the reaction were used (either unmodified or after correction to pH 5 judged by UI paper) as media in microplate experiments.

For comparison, hydrolysis was carried out on wheat straw (2 g) using concentrated H_2SO_4 (72%, 40 mL) at ambient temperature for 1 hour, then half was diluted to 10% acid v/v and autoclaved at 120 °C for 15 min. These samples were centrifuged (6000 rpm, 30 min), the supernatant decanted and run in HPLC.

2.3 Chapter 4 methods

2.3.1 Cultivation in 2L bioreactor

The bioreactor experiment was run in duplicate. Wheat straw hydrolysate (1600 mL) and deionised water (400 mL) were adjusted to pH 5 and a 2L bioreactor excluding pH and DO probes (Fermac 360, ElectroLab, UK) autoclaved for 15 min at 120 °C. The bioreactor was charged with the diluted hydrolysate under non-sterile conditions, and the pH and DO probes disinfected with ethanol. Antifoam A (2 mL) was added and the fermenter was aerated at 3L min^{-1} and stirred at 250 rpm for 30 min to fully saturate the medium with oxygen. The bioreactors (fitted with HCL (1 M) and NaOH (1 M) pumps) were used to adjust the pH to 4 then inoculated with 50 ml *M. pulcherrima* in YPD (OD_{600nm} = 0.6) under non-sterile conditions. The bioreactors were run at 25 °C, 250 rpm, pH 4, 2 L min^{-1} air for 3 days. The aeration rate was then lowered to 0.2 L min^{-1} and the agitation rate lowered to 100 rpm and run for a further 5 days. The pH and temperature were maintained throughout. Growth was monitored by OD_{600nm} and cell count and the sugar content monitored by HPLC.

2.3.2 Wheat straw hydrolysis – flow process

Samples of wheat straw hydrolysate were used as received from collaborator Lilia Zenker, Technical University of Hamburg, based on a published procedure.[327] In brief - temperature was varied from 150, 170 and 190 °C while other parameters were kept constant: flow rate (4 mL min^{-1}), biomass loading (20 g) and water to biomass ratio (10:1). The pressure was kept above 50 bar to assure water was still liquid. Temperature was controlled with an oven. Straw was loaded in a 50 mL fixed bed reactor and heated up to desired temperature. At this point, pre-heated water was passed through the reactor, cooled down after the reaction zone, depressurized and collected. After the required time, the water flow was stopped, the reactor cooled down, depressurized and the pretreated straw collected.

2.4 Chapter 5 methods

2.4.1 Analytical methods

Oligosaccharide detection was carried out using HPLC by collaborators at the University of York on Hewlett Packard Series 1100 with Evaporative Light-Scattering Detector with an Alltech 3300 Hi-Plex Na, 10 um, 300x7.7mm with a Hi-Plex Na, 10 um, 50 x 7.7 mm guard column. Mobile phase was 100% water, flow rate of 0.3 mL min^{-1}. This HPLC system was also used in the determination of rhamnose, levoglucosan, sucrose, fructose, mannose and galactose, using a normal phase Luna NH2 (5μm) column (Phenomenex, CA, USA) and Hi-Plex Pb (Agilent, CA, USA) both with acetonitrile and water, whereas furfural and HMF concentrations were found on reversed phase C18 column. The remaining sugars (glucose, xylose, arabinose and cellobiose) were analysed in Bath as per section 2.1.2. CHN and ICP-MS analysis was conducted on samples of 1:20(0) 190 – 210 °C in duplicate by collaborators at the University of York on samples of hydrolysate from which water was removed at 105 °C.

2.4.2 Microwave liquid hot water hydrolysis of lignocellulose

Wheat straw, seaweed, rapeseed meal or jellyfish pellets were combined with deionised water at the ratios specified (6 or 12 g wheat straw) in stirred 100 ml microwave pressure tubes, then heated to the temperatures specified in a MARS 6 microwave reactor (CEM, NC, USA). The vessels took between six and eight minutes to reach the set temperature, which was measured using a combination of IR and fibre optic detector, (Appendix 1conducted by collaborators at the University of York). Solid and liquid fractions were separated by filtration (Whatman 1), the solid fraction dried in an oven at 80 °C for five days then weighed, and the liquid fraction (henceforth referred to as the hydrolysate)

frozen at -20 °C until required. The fraction solubilised was determined by subtracting the mass of the dried solid fraction from the mass of straw added.

2.5 Chapter 6 methods

2.5.1 Composition of *M. pulcherrima* cells

Protein content: the nitrogen content of freeze-dried yeast cells was found by CHN analysis multiplied by the nitrogen to protein conversion factor 6.25, as per standard procedures.[328, 329]

Ash content: Weighed samples were combusted at 3000 °C in triplicate and the remainder re-weighed.

Lipid content: see above.

Carbohydrate content: Remaining mass.

2.5.2 Surface tension measurement

The surface tension of *M. pulcherrima* oil was measured at 22 °C using pendant drop tensiometry (Drop Shape Analyzer – DSA100E, Krüss, Germany) using the following equations (Figure 2.2).

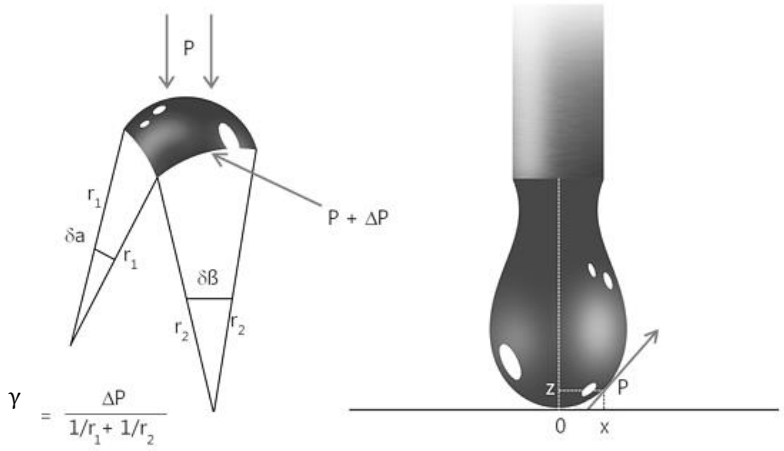


Figure 2.2. Surface tension, γ, via pendant drop tensiometry. The difference between the internal and external pressure, P, and the radii of the curvature of the surface are related by the Young-Laplace equation.

Surface/interfacial tension in all other cases was measured at 23 °C by a tensiometer (Attension Sigma 700, Biolin scientific, Sweden) using a flamed platinum Du Nuoy ring of a known radius. The surface tension, γ, was given by:

$$\gamma = \frac{\beta F}{4\pi R}$$

where F is the pull on the ring, R is the mean radius of the ring and β is a correction factor. For surface tension measurements the starting position of the ring was above the surface in the air, whereas for interfacial tension measurements it was in the aqueous phase below the oil and pulled up through the interface.

2.5.3 Soap synthesis

Rapeseed oil and sodium hydroxide were combined at an approximately 1:1 molar ratio, heated and stirred in a round-bottomed flask at 60 °C overnight. Reaction completion was confirmed by 1H NMR.

2.5.4 Emulsion preparation and creaming index measurement

The composition of the emulsions was that of chapter 5, table 5.3. All emulsions were diluted as required with DI water and included preservatives 0.1% EDTA and 1% 2-phenoxyethanol. Soap and oil were heated together to mix them. Aqueous phase components were mixed together by shaking then the warm oil phase added. Emulsification of these mixtures was with a homogeniser at 4000 rpm for 2 minutes at atmospheric pressure. The resulting emulsions were stored at room temperature. The total height and height of the aqueous phase to the lowest part of the interface was measured with digital callipers. The creaming indices for the emulsion were compared to the 'creaming' indices for the same volume of oil on water.

2.5.5 Particle size and polydispersity index

Samples were analysed by a dynamic light scattering particle size analyzer (Mastersizer 2000, Malvern Instrument Ltd, UK). The sample solution was dispersed in distilled water at 1800 rpm until an obscuration rate of 0.2 ±0.01% was obtained. Background measurements were taken for five scans over ten seconds and subtracted using an identical scanning method from the sample measurement. Data was analysed using the Mastersizer software using a polydisperse model. Droplet size measurements are reported as the volume-weighted mean diameter. The polydispersity index (PDI) of the emulsions was determined by the following equation, whereby d = the Z-mean, σ is the standard deviation of the z-mean

$$PDI = \left(\frac{\sigma}{d}\right)^2$$

Dynamic light scattering measures the fluctuation in intensity of light scattered by the particles as they undergo Brownian motion. The fluctuation in intensity gives the coherence time, T_c, which in turn enables derivation of the auto correlation function g_1 – a function that decreases with delay time τ. For a monodisperse emulsion a plot of $\ln(g_1)$

versus τ gives a straight line with a slope which is related to the diffusion coefficient D. From D the droplets radius, R, can be calculated using the Stokes-Einstein equation.

$$D = \frac{kT}{6\pi\eta R}$$

where k is the Boltzmann constant (J K^{-1}), T the absolute temperature (K) and η the viscosity of the medium (Pa s).

3 Development of *M. pulcherrima* as an oleaginous yeast for industrial cultivation on lignocellulose

3.1 Preamble

M. pulcherrima has been described previously as sometimes existing as a "pulcherrima" cell type with a spherical shape, thick walls and a droplet of lipid.[78, 314] These pulcherrima cells appear to be transitory between vegetative cells and spores then produce asci, which coincides with the oil droplet reducing in size, presumably as the lipid provides energy for asci formation. Firstly, it was necessary to establish whether *M. pulcherrima* could be induced to consistently behave oleaginously, producing a lipid content of over 20% cell dry mass. Logically, immediately before sporulation the lipid yield would be the highest. If sporulation is halted at this point, the yeast harvested should be oil-rich pulcherrima cells. Sporulation was previously induced in *M. pulcherrima* kept at 16 °C on dilute media, with sporulation occurring between 10 and 15 days.[86] Temperature and nutrient concentration were therefore selected as methods of controlling sporulation.

A useful attribute of a biotechnological microorganism is having the flexibility to grow on a range of carbon sources. *M. pulcherrima* was known to assimilate and ferment glucose,

and in some strains galactose. Also assimilated (but not fermented) are sorbose, maltose, sucrose, cellobiose, trehalose, melezitose, ribose, adenitol, sorbitol, succinic and citric acid.[86] Xylose has also been shown to be metabolised by *M. pulcherrima* and used to form lipid.[143] This is a particularly useful characteristic in the upgrading of lignocellulosic hydrosylates. However, strains used by Pitt and Miller[86] did not assimilate arabinose or lactose, and Papanikolaou[95] found that although *M. pulcherrima* assimilated glycerol, the lipid content produced was only 1.5% cell dry mass.

Although used as a post-harvest pest-control agent known to outcompete other yeast species, through production of pulcherrimin and subsequent iron sequestration from the media, the ability of *M. pulcherrima* to grow and outcompete other microorganisms in liquid media has not been investigated.[316, 330, 331] Nor have studies been published of *M. pulcherrima* grown at larger scale or in controlled bioreactors. The concept of using *M. pulcherrima* as an organism for an industrial cultivation under non-sterile conditions was thus evaluated.

The production of second-generation fuels through the fermentation of lignocellulose is hampered by the high cost of lignocellulose depolymerisation and the production of toxic inhibitory side products often inhibit the microorganisms used to ferment the hydrolysate later in the process.[279, 332] The tolerance of yeast species to these inhibitors varies, and some oleaginous yeasts such as *T. cutaneum* have demonstrated high tolerance – its growth being reduced by 50% by 4.7 mM furfural.

The conversion of lignocellulose to microbial products directly, without the need for additional enzymes (consolidated bioprocessing, CBP), has attracted much interest.[248] Originally filamentous fungi, such as *Trichoderma reesei*, were investigated due to their ability to degrade lignocellulosic waste through the production of cellulolytic enzymes, assimilation of several substrates for growth and a very high resistance to inhibitors.[333] Currently a range of organisms are being developed that can produce ethanol from the insoluble components of cellulosic biomass. One promising candidate for CBP is *Pichia stipitis*, principally known as one of the few yeasts able to assimilate and ferment pentoses to ethanol.[334] Recently, its genome was sequenced, revealing the presence of many cellulases and hemicellulases, while recent studies have successfully managed to increase its inhibitor tolerance towards lignocellulosic by-products through UV mutagenesis.[335] Other yeasts considered for CBP are *Kluyveromyces lactis*, *Kluyveromyces marxianus* and *Hansenula polymorpha.* These strains, like *Pichia stipitis*, have a broader range of usable sugars than *S. cerevisiae* and also show a considerable thermotolerance, an important feature that can be used to maintain sterility in an industrial process.[334]

Some fungi, especially the Ascomycota phylum of which *Trichoderma reesei* and *M. pulcherrima* belong, produce enzymes that hydrolyse lignocellulose. *M. pulcherrima* has been reported to produce extracellular[336] and the more active intracellular[337] β-glucosidases, three types in total, active against (1 → 4)-β and (1 → 2)-β glycosidic linkages. *M. pulcherrima* was found not to produce β-D-xylosidase.[338] This gives *M. pulcherrima* the potential to be used in CBP, however, no reports exist detailing the culturing of *M. pulcherrima* on lignocellulose under laboratory conditions.

In this chapter, the oleaginous yeast *M. pulcherrima* was assessed in a CBP approach by firstly examining its behaviour on model feedstocks, then culturing the yeast on partially depolymerised wheat straw. The wheat straw was pre-treated to solubilise the various saccharides but had not undergone full depolymerisation to monosaccharide-rich feedstocks as used by most studies of oleaginous yeast on lignocellulose.

3.2 Carbon source utilisation

Given that there is a large variation amongst strains' ability to catabolise sugars, the capability of the strain of *M. pulcherrima* obtained from the National Yeast Culture Collection, Norwich, UK (see section 2.1.1) to assimilate sugars was assessed (Figure 3.1.) As well as the sugars in isolation, combinations of sugar were assessed, in order to examine more realistic models of lignocellulose hydrolysates and to examine glucose repression. It was expected that glucose would be the preferred substrate, as is the case for other yeasts.

M. pulcherrima can metabolise a large range of sugars, including pentoses, demonstrating that growth on the hemicellulosic fraction of hydrolysed lignocellulose is feasible (Figure 3.1). Unsurprisingly, considering *M. pulcherrima'*s preferred biological niche of fruit, the best growth was seen on fructose. Glucose alone gave the second best growth, as well as improving the growth of *M. pulcherrima* when other less favoured sugars were present. Given that no other single sugar, nor combination of sugars yielded as much biomass as glucose alone (aside from fructose), it is likely that glucose repression is leading to slower total sugar consumption – as glucose is first consumed and then the enzymes required to enable catabolism of the other sugars are enabled. Despite this, provided glucose was present, the growth was only slightly reduced compared to that on glucose alone for all sugars in the same fermentation time.

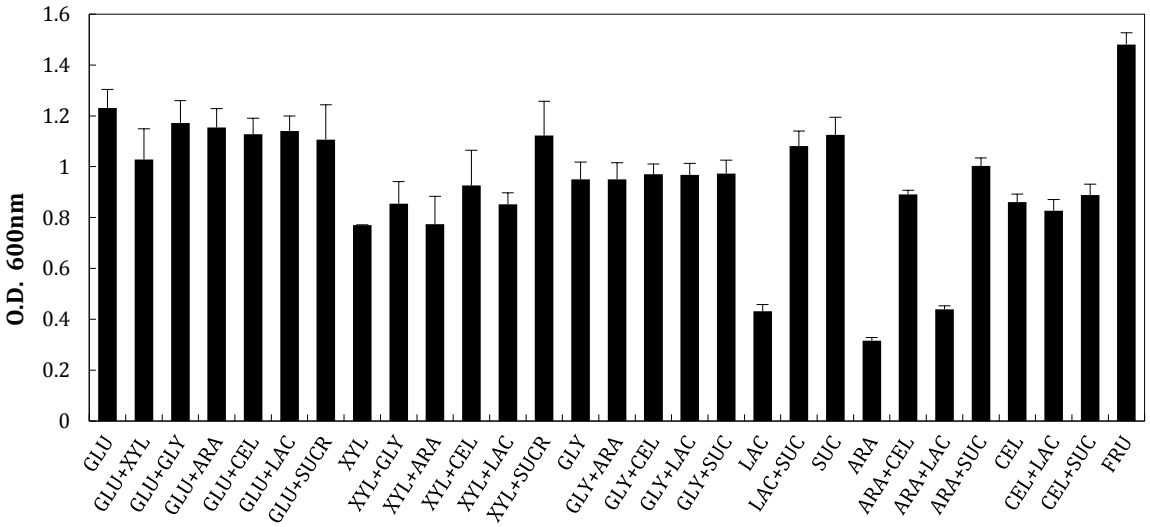


Figure 3.1 Biomass productivity of *M. pulcherrima* on a range of simple sugars at a concentration of 30 g L^{-1} . Optical density of the culture after 72 hours 25 °C, 180 rpm. GLU = glucose, XYL = xylose, GLY = glycerol, ARA = arabinose, CEL = cellobiose LAC = lactose, SUC = sucrose, FRU = fructose. [323]

Sucrose, a disaccharide of glucose and fructose, was assimilated, suggesting that *M. pulcherrima* can produce enzymes capable of hydrolysing this glycosidic bond. The poor growth on lactose corroborates Pitt and Miller's findings.[86]. These results agree with a study subsequently published by Boundy-Mills *et al.*[110] using the strain (UCDFST 11-1039). The exception as that their strain did not consuming arabinose at all, rather than just poorly. They also examined mannose, rhamnose and galacturonic acid, finding no growth on the latter two compounds, however these sugars were used at only 5 g L^{-1} concentrations and in isolation of any other sugar. The ability of *M. pulcherrima* to grow on multiple sugar sources, including pentoses, means that it can theoretically utilise a larger fraction of the sugars present in lignocellulose, and henceforth give greater yields of cell dry mass than alternative yeast species such as *S. cerevisiae*.

3.3 Promoting oleaginous behaviour

Initially *M. pulcherrima* was cultured on glycerol, potentially a waste product from the biodiesel production process. Glycerol is available on significant scale and was known to be catabolised by *M. pulcherrima*. The yeast was cultured over a range of temperatures and times in order to find conditions that gave the greatest yield over the shortest time (the lipid productivity). It was anticipated that lower temperatures, combined with nitrogen starvation would halt sporulation and in so doing accumulate a greater number of fat containing cells. The temperature was initially the same for all cultivations (25 °C)

to allow the yeast to divide rapidly and consume the nitrogen, then the temperature was lowered once the stationary phase was reached. This was carried out in a nutrient rich medium as used by Papanikolaou *et al.*[95]

The mass of the dried cells per unit volume of medium is defined as the cell dry mass (X, g L^{-1}) The lipid content (%), is the percentage of the cell dry mass that consists of lipid. The amount of lipid present in the medium is the lipid yield (LY, g L^{-1}). Lipid productivity is the amount of lipid produced over time (g L^{-1} d^{-1}). Figure 3.2 indicates that the fermentation temperature is inversely correlated with lipid content with the maximum lipid content for 26 % attained at 15 °C. This supports the hypothesis that decreasing the temperature halts sporulation and the cells convert the excess carbon source (glycerol) into lipid.

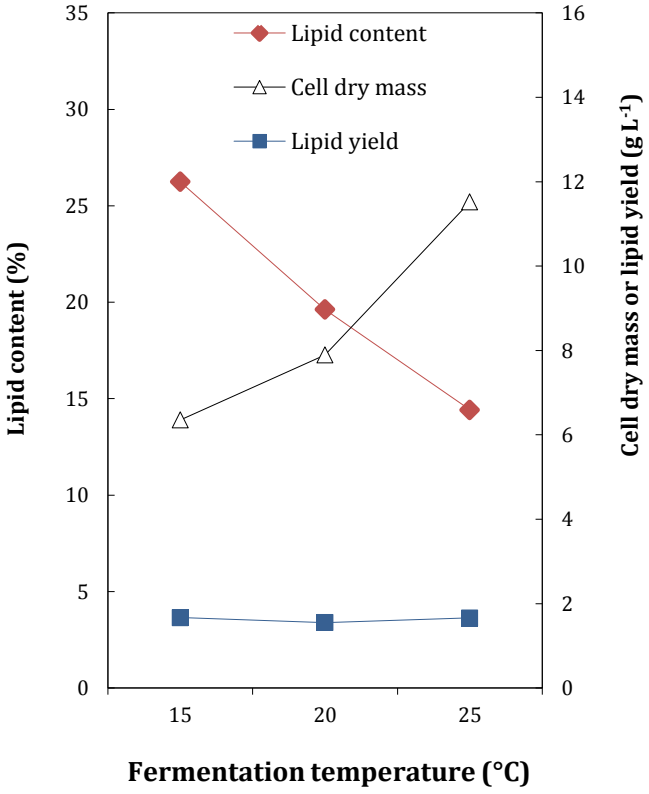


Figure 3.2. Effect of initial temperature on lipid content (%), cell dry mass, and the lipid yield of *M. pulcherrima* grown on nutrient rich glycerol medium for three days at 25 °C then twelve days at the stated temperature at 180 rpm throughout.

In terms of generating the maximum cell dry mass, a high initial temperature is beneficial as in this nutrient-rich medium fermentation temperature correlates directly with cell dry mass. This indicates that the cell dry mass is still increasing when the temperature was lowered after three days, from 25 °C to the stated temperature and this process is unsurprisingly slower at lower temperatures (the OD of the culture at 15 °C increased by only 0.1 over 8 days). The inverse correlation between lipid content and cell dry mass

means that despite the increase in lipid content, the lipid yield does not vary significantly with temperature in this experiment – yielding 1.67, 1.55 and 1.66 g L^{-1} at 15, 20 and 25 °C respectively. This suggests that *M. pulcherrima* can grow at a wider range of temperatures than other yeasts, possibly allowing outdoor cultivation. The maximum lipid productivity was 0.11 g L^{-1} d^{-1}. This is five time the lipid productivity obtained by Papanikolaou *et al.*[95] when culturing *M. pulcherrima* on glycerol. However this value is still at the lower end of the range of lipid productivities obtained by oleaginous yeasts, the maximum being 14.16 g L^{-1} d^{-1} obtained by *C. curvatus* cultured under fed batch conditions on glycerol.[161] Consequently, it was concluded that optimisation was necessary.

As a dilute, nutrient-poor medium has also been reported to prevent sporulation,[86] a nutrient-poor, and also lower cost Yeast Minimal Medium (YMM, see section 2.1.1) was developed by reducing the individual nutrient concentrations and comparing the growth of *M. pulcherrima* on these media.[323] YMM was used to ferment *M. pulcherrima* over a range of times and the lipid content and cell dry mass were determined (Figure 3.3).

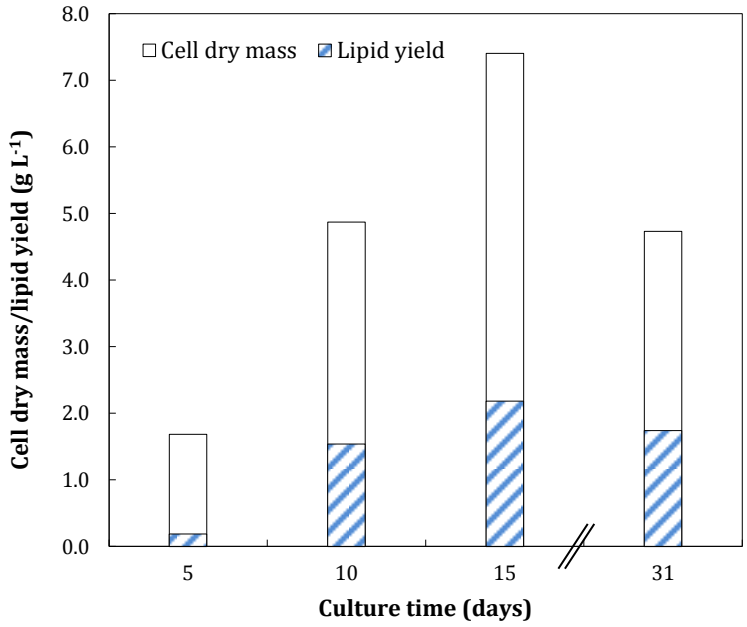


Figure 3.3. Effect of time on cell dry mass (white bars) and lipid yield (shaded blue area) of *M. pulcherrima* cultured on YMM with glycerol. Grown in 250 ml aliquots in 500 ml conical flasks being shaken at 180 rpm. Day 5 was held at 15 °C throughout, other samples grown at 25 °C for 3 days, then 15 °C for the remaining time.

The maximum lipid content (47%) was found after an initial temperature of 25 °C (Figure 3.3). This is clearly in the oleaginous range, showing that *M. pulcherrima* can produce significant quantities of lipid on glycerol under optimised conditions. This corroborates the hypothesis as lipid appears to be accumulating with time, then after extended periods

of time (31 days), cell death, sporulation or carbon starvation leads to a lower cell dry mass and lipid content. The nutrient-poor minimal medium was effective in increasing the lipid content, suggesting that the nitrogen content in the original medium was too high, or that additional stress from the lack of nutrients is beneficial. The greater maximum lipid yield of 2.92 g L^{-1} meant that the lipid productivity was higher at 0.20 g L^{-1} d^{-1}, if still lower than the median value from the literature (0.60 g L^{-1} d^{-1}.)

3.4 Model lignocellulose hydrolysate

The carbon source type is one of the variables that can affect the growth and lipid content, and it was predicted that these responses would be different when grown on the carbon sources present in lignocellulose, rather than glycerol and glucose. Due to glucose repression, it is also expected that glucose will be consumed initially, then on its depletion the other sugars will be consumed. To assess the behaviour of *M. pulcherrima* on lignocellulose but isolating just the carbon source, sugars representative of lignocellulose hydrolysate (glucose, xylose, arabinose, cellobiose) were selected and combined at equal concentration by mass (7.5 g L^{-1}). The growth, lipid content, and rate of sugar consumption by *M. pulcherrima* when cultured on this mixture was compared to glycerol.

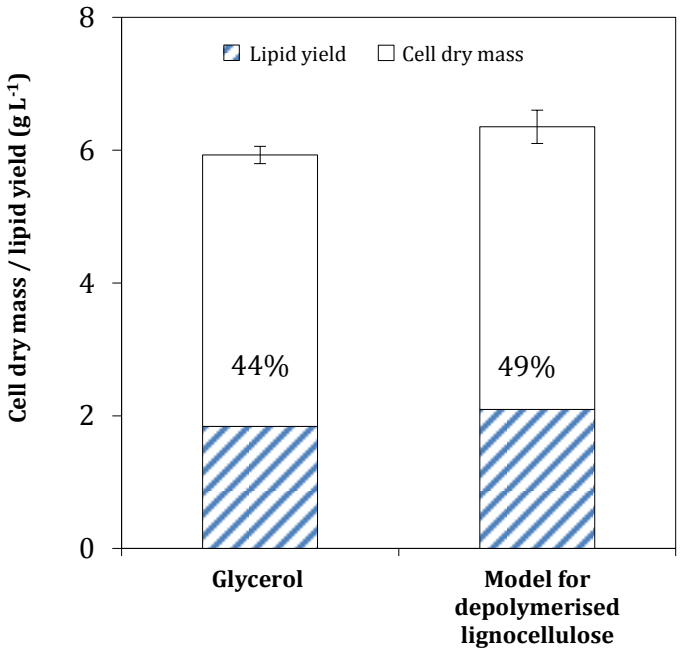


Figure 3.4. Dry cell weight, lipid yield and (lipid content %) of *M. pulcherrima* cultivated for 10 days on glycerol and model lignocellulose (7.5 g L^{-1} of each of glucose, xylose, arabinose and cellobiose) with nutrients of YMM at 25 °C and 180 rpm.

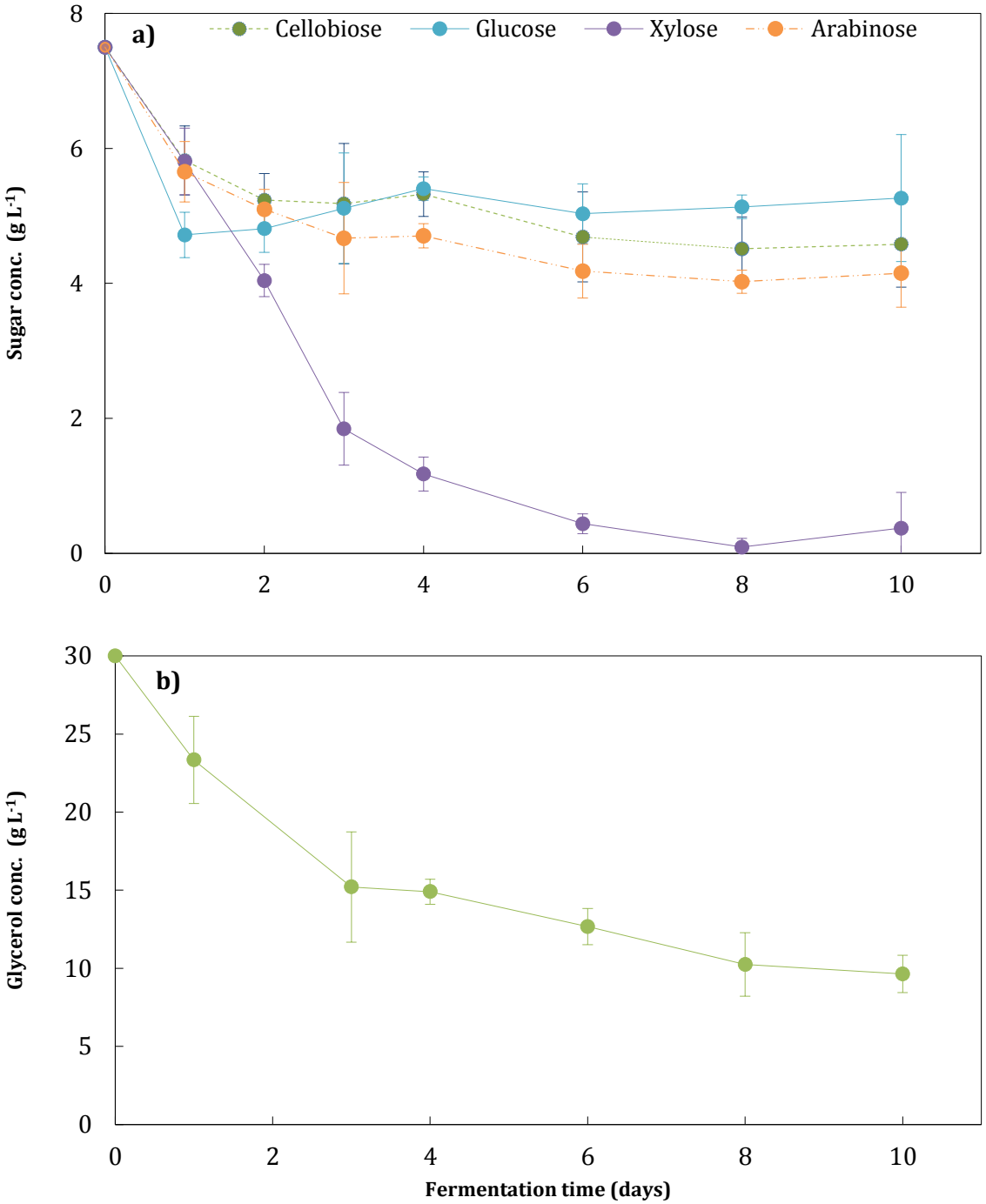


Figure 3.5. ***M. pulcherrima*** **cultivated for 10 days on glycerol and model lignocellulose as per Figure 2.4 at 25 °C and 180 rpm. a) consumption of sugars in model lignocellulose; b) consumption of glycerol.**

M. pulcherrima produced similar amounts of biomass and lipid on the model lignocellulose as it did on glycerol, with lipid contents of 49% and 44% respectively. Significantly, all four sugars of the model feedstock were starting to be catabolised by *M. pulcherrima* within the first day, and were rapidly consumed for the first three days. By day three the culture had entered the stationary phase (data not shown), and the consumption of

glucose, arabinose and cellobiose practically halted. Interestingly, consumption of xylose was more rapid than the other sugars until its approximate exhaustion after eight days. It is possible that *M. pulcherrima* catabolises xylose directly, or that xylose is converted into an unknown secondary product that is not detected on the HPLC.

The ability of *M. pulcherrima* to grow and produce lipid on the model lignocellulose hydrolysate was confirmed, yet the lipid yield and lipid productivity were low at 2.09 g L^{-1} and 0.21 g L^{-1} $d^{-1,}$ respectively. The repeated-batch method is known to increase the lipid yield of oleaginous yeast cultures, with most of the published experiments yielding the highest concentration of lipid (from 5 g L^{-1} up to 78 g L^{-1}) using this approach.[90, 166, 178, 239] In addition, yeast extract and vitamins such as biotin are expensive and would not be suitable for use on the large scale production of a palm oil substitute. *M. pulcherrima* was therefore cultured in non-sterile, repeated-batch fermentations with and without some nutrients (Table 2.1) and fed with the sugar composition in model lignocellulose (Figure 3.6).

	With biotin	Without biotin	With yeast extract	Without yeast extract
Yeast extract	-	-	+	-
Biotin	+	-	-	+
Additional ammonium chloride	-	-	-	+

Table 3.1. Nutrient composition of fed-batch media. + = nutrient included, - = nutrient absent.

The repeated-batch method successfully increased the lipid yield to a maximum of 6.04 g L^{-1} (with yeast extract), as the cell dry mass for these conditions increased to 17.3 g L^{-1} despite the lipid content decreasing to a maximum of 35%. *M. pulcherrima* was similarly productive in terms of lipid content and lipid yield, with the exception of the medium with biotin, on all media, indicating that supplemental nutrients may not be essential. It appears that the reduction in nitrogen content arising from removal of yeast extract increases the lipid content (39%), but reduces the cell dry mass as lipid production is stimulated but protein production (necessary for growth) is limited. Biotin was found to be a less effective nutrient than yeast extract.

This demonstrates that *M. pulcherrima* can produce 15 g L^{-1} dry cell mass with a lipid content of 33% even with no biotin present. The ability to produce lipid at a rate of 151 mg L^{-1} d^{-1} in absence of a vitamin supplement could have an extremely positive impact on the cost of a putative industrial process

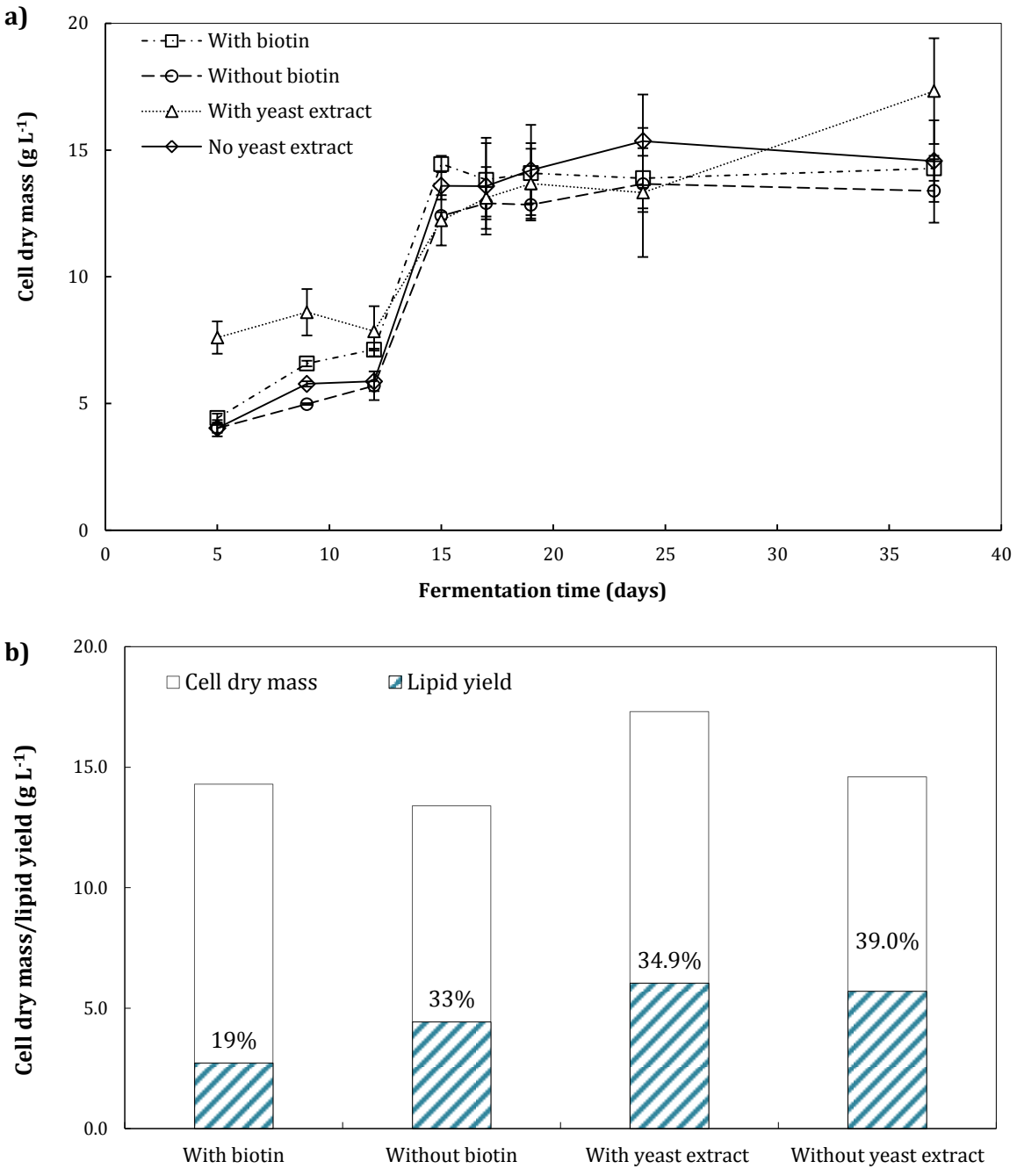


Figure 3.6. a) Cell dry mass and b) lipid yield and (lipid content %) from fed-batch culture of *M. pulcherrima* in YMM with supplemental nutrients as per and 7.5 g L^{-1} of glucose, xylose, cellobiose and arabinose. Cultured in non-sterile conditions at 25 °C, 180 rpm shaking, and fed with additional sugar every 3 days until 24 days, then kept at 15°C, 0 rpm for 12 days.

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3.5 Demonstration of *M. pulcherrima* at 500 L scale

Temperature had little effect on the lipid yield and the optimum time of 15 days had been determined. The ability of *M. pulcherrima* to produce lipid under low-cost and hostile conditions (i.e. non-sterile, minimal temperature control, nutrient-poor medium) had not been examined. As discussed above, *M. pulcherrima* is known to produce pulcherrimin, which chelates and thus depletes iron from the surroundings, hence inhibiting the growth of other yeast and bacteria.[316, 318, 330, 331] Raceway ponds have been used as a low-cost system for microalgae cultivation. The simple construction and lack of containment or process control means that the capital and running costs are lower, however contamination with other species of algae is problematic.[49, 297]

To examine the suitability of *M. pulcherrima* to be cultured in low-cost non-sterile conditions, the yeast was cultured in two 500 L raceway ponds agitated by a paddle wheel at 10 rpm, using 30 g L^{-1} glycerol in YMM, in a temperature controlled glasshouse (Figure 3.7). The pH was initially set to 4 to discourage microbial growth, then manually adjusted using dilute potassium hydroxide and hydrochloric acid to maintain an optimum pH between 3-5. The pH, OD (corresponding to cell dry mass), and temperature were measured daily (Figure 3.8).

Figure 3.7. *M. pulcherrima* cultivated at 500 L scale in raceway ponds (left), settling on paddle wheels (middle) and close up view (right).

The pH was recorded and after an initial increase, the yeast seemingly started to excrete acids into the media in order to lower the pH. However, the yeast also produces CO_2 which could form carbonic acid reducing the pH further. Presumably due to the low concentration of the yeast at first (500 ml inoculum in YMS, 48 hrs, 25 °C), some bacteria was observed via flow cytometry over the first three days. Excitingly, once the yeast was established and had reduced the pH to 3, only *M. pulcherrima* was observed from this point on, demonstrating the efficacy of *M. pulcherrima* to be grown axenically under non-sterile conditions. It is not clear whether it outcompeted the bacteria using iron

sequestration or by just lowering the pH, but the former was found to be more effective at lower pH.[316]

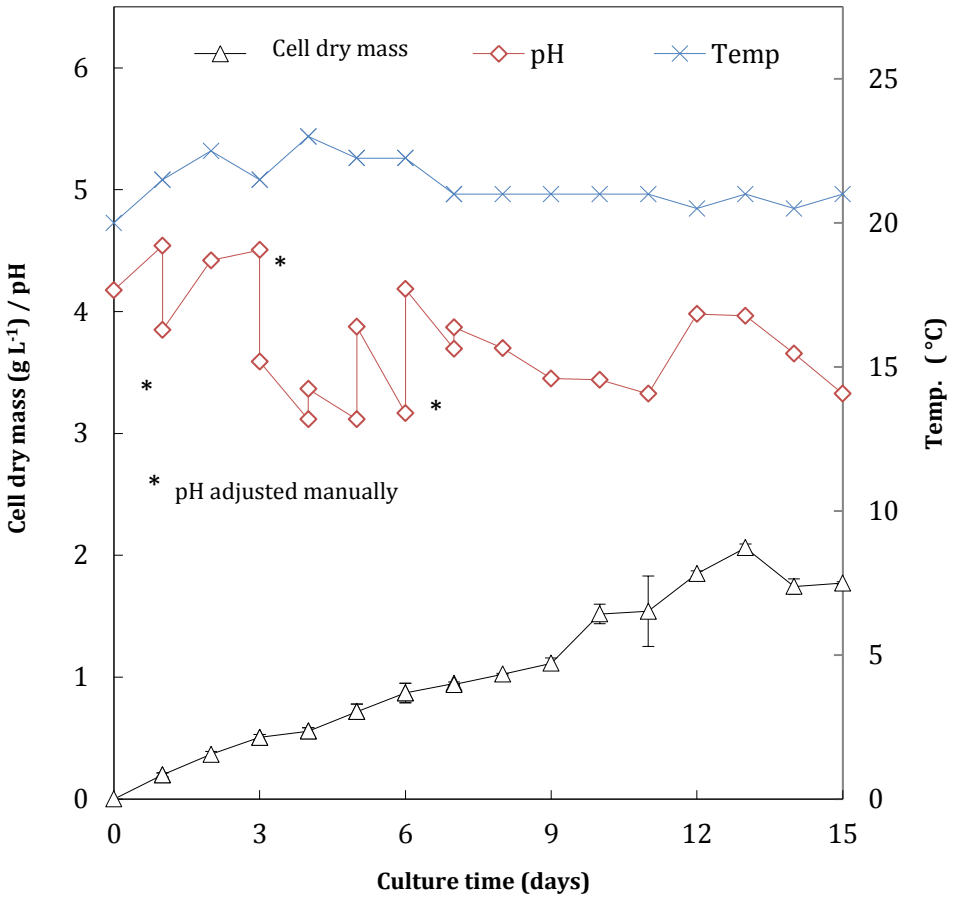


Figure 3.8. Cell dry mass, pH and temperature of *M. pulcherrima* cultures grown on glycerol in raceway ponds.

Agitation was an issue, which led to poor gas transfer in the system. *M. pulcherrima* is a flocculating yeast and significant quantities stuck to the bottom of the reactor and to the paddle wheels (Figure 3.7), so may not have received enough oxygen and thus growth and lipid production would have been slower than under ideal conditions. This was reflected in the low density of biomass found (2 g L^{-1}) and also the optical density near the surface was lower than in the bulk (0.9 g L^{-1}). Despite this, a reasonably high lipid content of 35% was produced. The bioreactor type used here is not optimised for yeast fermentation, yet still demonstrates that *M. pulcherrima* is impressively versatile and can grow and produce lipid under non-sterile conditions.

The oil produced by this experiment was analysed by 1H NMR and the triglyceride portion, esterified and analysed by GC-MS (Figure 3.9). The oil contained around 90 wt% triglycerides and 10 wt% sterol. The fatty acid profile was relatively diverse, though the majority of the fatty acids were unsaturated C18, similar to rapeseed oil. Significant

quantities of a sterol were found (the alpha-protons can be seen at δ3.5 ppm), the sterol was presumed to be ergosterol, which is commonly found in yeast cell membranes.[339] No aromatic compounds were detected in the oil.

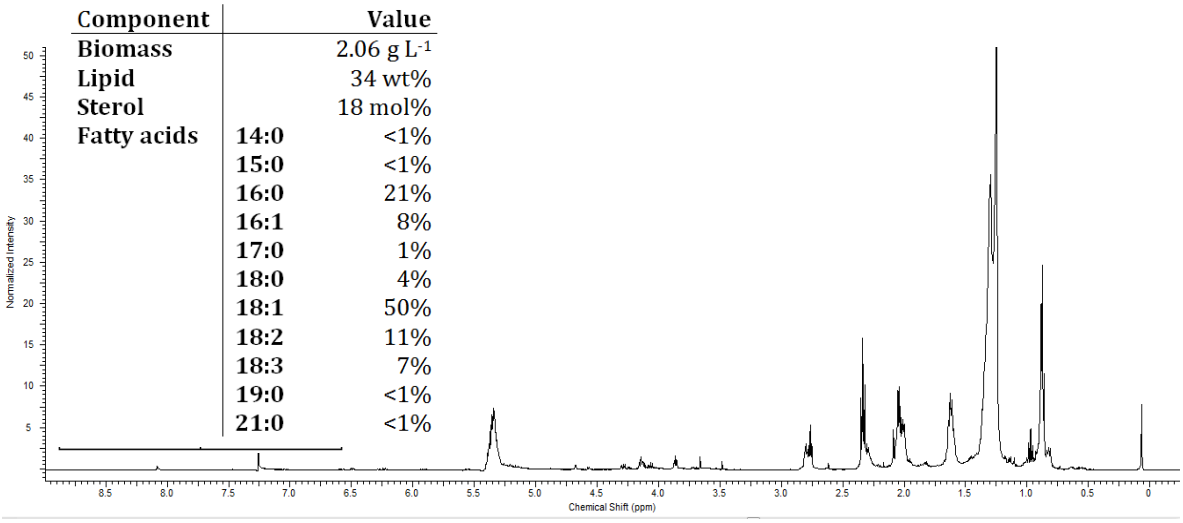


Figure 3.9. ^{1}H NMR and FAME (fatty acid methyl ester) profile of oil from *M. pulcherrima* grown in raceway pond, un-purified.

3.6 Properties of the lipid produced by *M. pulcherrima*

In order to examine the oil formed in more detail, a 1 L scale culture grown on glycerol on YMM in 2 x 500 ml shake flasks was fermented at 28 °C for 6 days then 15 °C for 6 days, the dried biomass collected and the oil extracted. The FAME profile, NMR spectrum, energy density and dynamic viscosity of this oil were measured.

These values are reasonably similar to palm oil. The lipid content was slightly lower than observed in previous experiments, probably due to using a non-optimised oil extraction technique. Despite the FAME profile being more similar to rapeseed oil, the viscosity of *M. pulcherrima* oil was approximately ten times that of palm oil, suggesting it could provide the thickening properties necessary in far lower quantities. The higher viscosity and lower energy density may be due to the presence of ergosterol, not seen in NMR of *M. pulcherrima* oil when cultured on glucose.

Parameter	Value for *M. pulcherrima* oil	
Optical density	19.71 ±0.25	
Cell count (m cells m^{L-1})	4.57 ±0.34	
Dry biomass (g L^{-1})	5.0	
Oil content (%)	26 ±2%	
	M. pulcherrima oil	Palm oil
Density (g cm^{-3})	0.76 ±0.16	0.91 g cm^{-3}
	(at 60 °C)	(at 40 °C)
Dynamic Viscosity at 40 °C (Pa s)	0.58 ±0.06	0.050
Energy density (MJ kg^{-1})	27.33	37.6

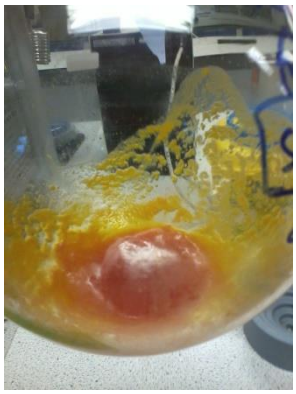


Table 3.2 Properties of *M. pulcherrima* culture and the oil extracted from it (pictured right), and properties of palm oil. Palm oil values were taken from Cadena *et al.*[340] Cell count obtained by flow cytometry, which also showed no particles of bacterial size.

3.7 Tolerance of *M. pulcherrima* to inhibitors

Another key issue with the fermentation of lignocellulose is the presence of inhibitors. The pre-treatment and harsh conditions of hydrolysis used on the lignocellulosic feedstocks, such as wheat straw, can cause the degradation of the released sugars into furfural, 5-hydroxymethylfurfural (5-HMF) and organic acids. The lignin component can also degrade partially into a range of monomeric aromatic compounds: vanillin, vanillic acid, 4-hydroxybenzoic acid (4-HBA), syringaldehyde, coniferyl alcohol.[35] In order to establish the tolerance of *M. pulcherrima* to non-detoxified lignocellulose hydrolysates, pure inhibitor compounds were added to YMM containing 30 g L^{-1} glucose. Typical low, medium and high concentrations of inhibitors were used (Table 2.3), in accordance with previous published reports.[35, 275, 290-292]

Inhibitor	Low value		Medium value		High value	
	(mM)	(g L^{-1})	(mM)	(g L^{-1})	(mM)	(g L^{-1})
Furfural	1	0.10	10	0.96	60	5.76
5-HMF	1	0.13	10	1.26	60	7.57
Acetic acid	10	0.60	60	3.60	200	12.01
Formic acid	10	0.46	60	2.76	200	9.21
Levulinic acid	10	1.16	60	6.97	200	23.22
Vanillin	1	0.15	10	1.52	30	4.56
Vanillic acid	1	0.17	10	1.68	30	5.04
4-HBA	1	0.14	10	1.38	30	4.14
Syringaldehyde	1	0.18	10	1.82	30	5.47
Coniferyl alcohol	1	0.18	10	1.80	30	5.40

Table 3.3. Concentrations of inhibitors used to test the tolerance of *M. pulcherrima*

The effect of the inhibitors was analysed using a 96 well plate format with cultures maintained for a minimum of seven days by optical density. To correct for absorption of light at 600 nm by the inhibitors, the increase in OD from the time at which the cultures were inoculated was determined and the data were normalised to the control medium which had no inhibitor present, to allow for comparison across the plates. Initially *M. pulcherrima* was cultured at 25 °C without increasing the pH in order to simulate an industrial process that did not involve post-hydrolysis pH modification.

Growth of *M. pulcherrima* was not significantly inhibited by most compounds tested, except at high concentrations. For example, furfural and 5-HMF generally substantially affect the growth of most yeasts at concentrations of 10 mM or lower.[278] When furfural concentrations were inhibitory, growth was slower, with longer lag times (data not shown). The organic acids (acetic, formic and levulinic) at 10 or 60 mM concentrations increased the growth of *M. pulcherrima*, but were inhibitory at 200 mM. Acetic acid was particularly toxic at this concentration.

Of the aromatic alcohols, acids and aldehydes produced from the decomposition of lignin, vanillic acid and 4-hydroxybenzoic acid (4-HBA) had little effect on growth, though vanillin and syringaldehyde were inhibitory except at low concentrations (1 mM, 0.15 g L^{-1}). Coniferyl alcohol was also inhibitory at the high 30 mM concentration.

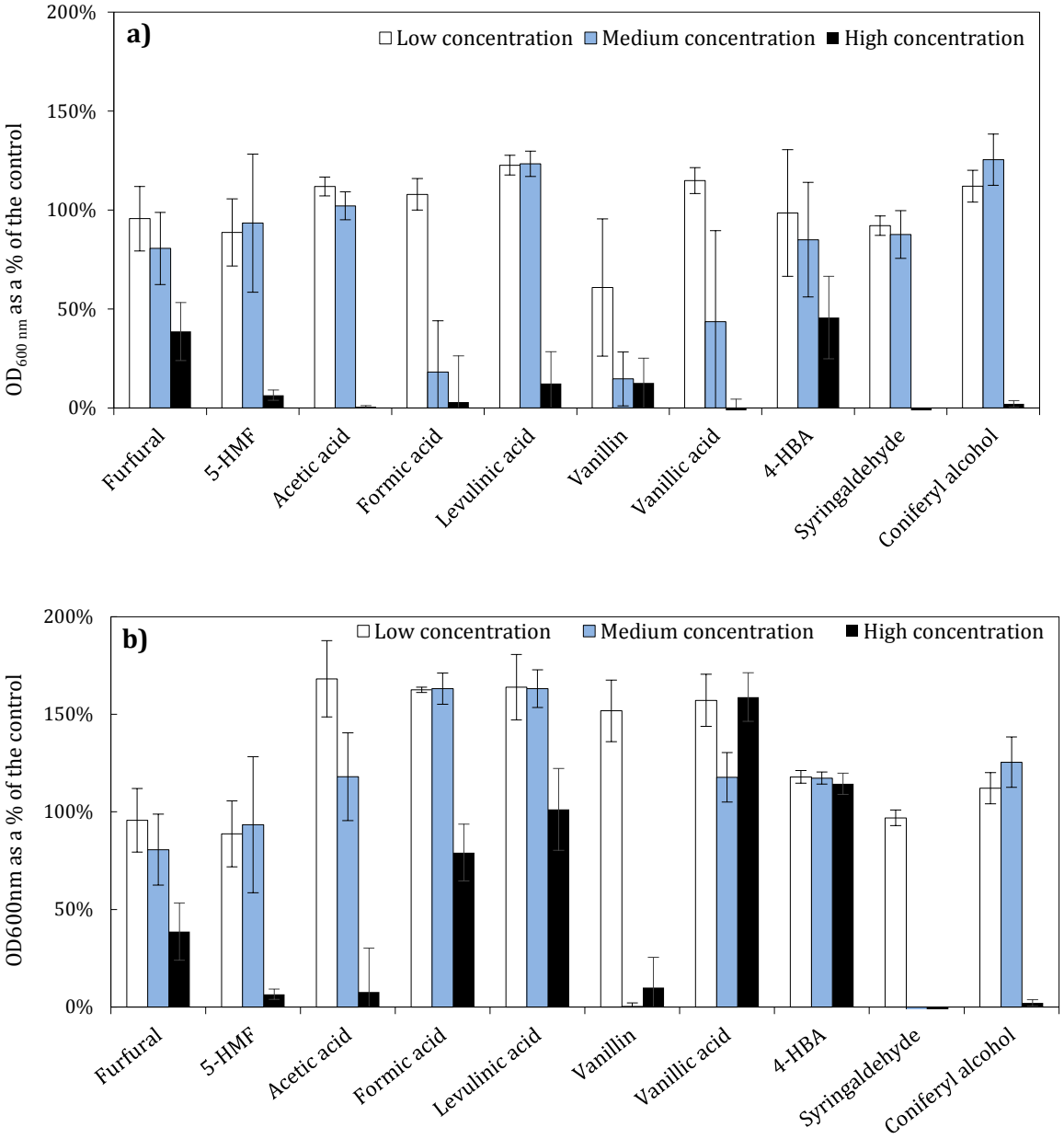


Figure 3.10. Increase in optical density of *M. pulcherrima* cultured in 96-well microplates over seven days cultivation in minimal medium dosed with inhibitors at the concentrations given in Table 3.3. The plates were cultured (25 °C, 180 rpm) at a) without pH modification b) adjusted to pH 5.

Sitepu *et al.*[110] later confirmed these results, finding that their strain of *M. pulcherrima* UCDFST 11-1039 grew on furfural at up to 1 g L^{-1}, acetic acid at 2.5 g L^{-1} and HMF at up to 2 g L^{-1}. This work therefore covers a more extensive range of concentrations and inhibitor compounds.

High concentrations of syringaldehyde, vanillic acid and 4-HBA were partially soluble in the medium with unadjusted pH, and so the lack of increase in OD for these results is likely to be caused by light being scattered by particles of these compounds rather than the

absence of yeast cells. It was thought that the addition of base, when raising the pH to 5, would deprotonate the acids and help them dissolve, which was correct in the case of 4-HBA and vanillic acid. The other acids also (acetic, formic and levulinic) gave surprisingly poor results at high concentrations of the acids in the non-pH adjusted media given that *M. pulcherrima* is a reported acidophile. The increase in pH allowed *M. pulcherrima* to grow far better on these media as the pH is closer to the optimal value.

In actual lignocellulosic hydrolysates multiple inhibitors will be present simultaneously. The effect of combinations of inhibitors on *M. pulcherrima* was therefore tested. For example, while alone *Pachysolen tannophilus, Scheffersomyces stiptis*, and *Escherichia coli* have good tolerance to single inhibitors this is reduced dramatically when acetic acid is used in conjunction with the furfurals.[35, 276, 284] To examine what effect this combination might have on *M. pulcherrima*, the medium concentration value of acetic acid was combined with the medium concentration value of all other inhibitors (Table 3.4).

Inhibitor	Inhibitor conc.		Acetic acid concentration	
	(mM)	(g L^{-1})	(mM)	(g L^{-1})
Furfural	5	0.48	30	1.8
5-HMF	5	0.63	30	1.8
Formic acid	30	1.38	30	1.8
Levulinic acid	30	3.485	30	1.8
Vanillin	5	0.76	30	1.8
Vanillic acid	5	0.84	30	1.8
4-HBA	5	0.69	30	1.8
Syringaldehyde	5	0.91	30	1.8
Coniferyl alcohol	5	0.9	30	1.8

Table 3.4. Combinations of acetic acid and inhibitors for tolerance of *M. pulcherrima*.

The growth of *M. pulcherrima* on combinations of acetic acid and inhibitors was approximately equal to the control medium for all combinations of inhibitor (Figure 3.11). That the most strongly inhibiting combinations of acetic acid and furfurals did not affect *M. pulcherrima* suggests it may be highly suitable for growth on genuine hydrolysates.

Unless depolymerised under extremely harsh conditions, such as high loadings of sulfuric acid at elevated temperatures and pressures, lignin is not broken down and although solubilised, remains relatively intact in the fermentation broth. We therefore tested the

impact of lignin on the growth of *M. pulcherrima*. The experiments were carried out in 50 ml falcon tubes, at 22 °C and stirred at 180 rpm. The OD_{600nm} and pH were measured.

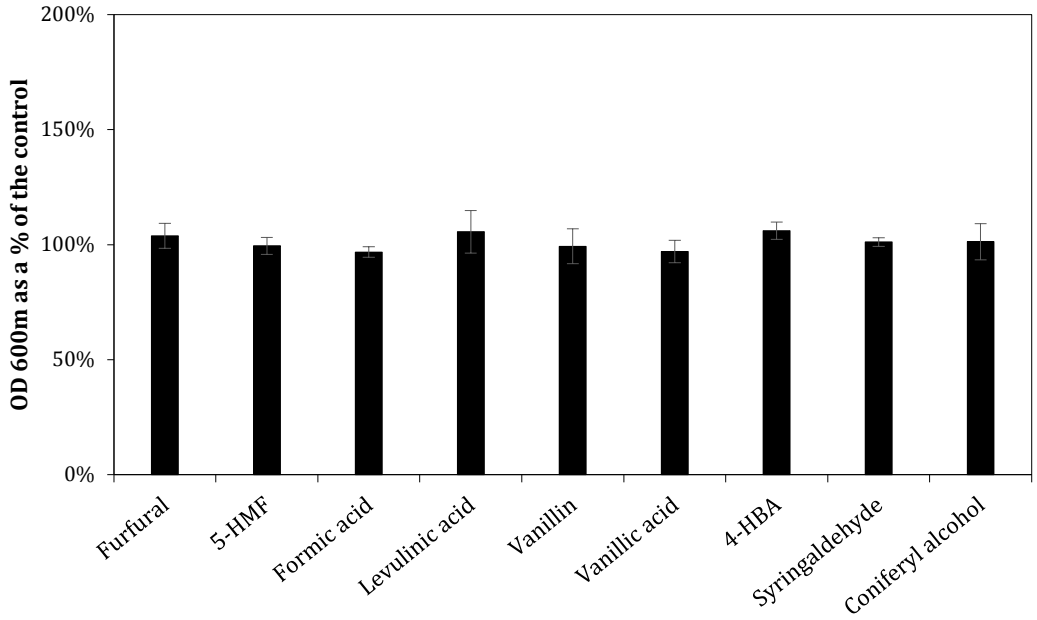


Figure 3.11. Increase in optical density of *M. pulcherrima* cultured in 96-well microplates over seven days fermentation in minimal medium dosed with inhibitors at the concentrations specified in Table 3.4. The plates adjusted to pH 5 and held at 25 °C with shaking at 180 rpm.

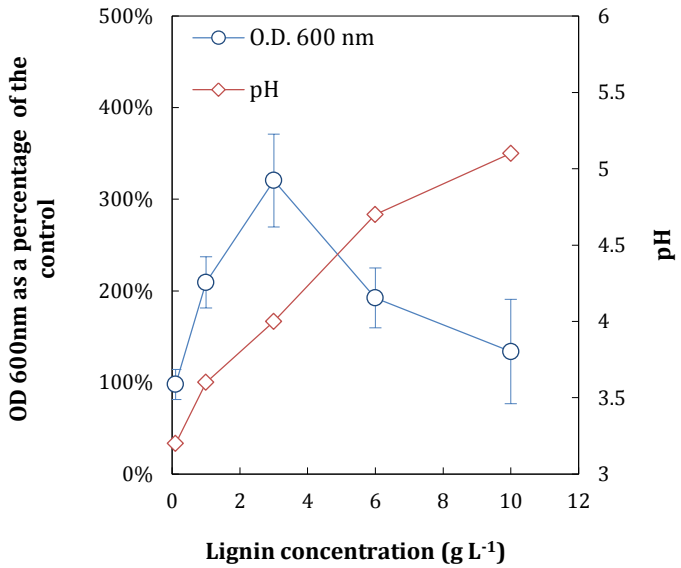


Figure 3.12 Optical density and final pH of cultures of *M. pulcherrima* after 7 days growing in the presence of different concentrations of alkali lignin. The data is expressed as a ratio of the control medium stirred at 180 rpm at 22 °C.

The results on completion of the culture, normalised to the control medium, are shown in Figure 3.12. Firstly, the presence of lignin in the broth was found not to interfere with the accuracy of determining growth rates via optical density. Lignin, even present at 10 g L^{-1}, in the fermentation broth does not have an inhibitory effect on the growth of *M. pulcherrima*. At lower concentration the cell count and OD_{600nm} is vastly increased when compared to the control. The most likely cause of this increase in growth is that the addition of alkaline lignin to the broth increased the pH from 3.2 (sub optimum), to 5.1. Previously the optimal growth for *M. pulcherrima* has been observed to be between pH 3.5 - 4.5, which coincides with a lignin concentration of 3 g L^{-1} in this experiment.

Overall, it appears that provided the inhibitor concentration is moderate, and ideally if the pH is increased to 5, *M. pulcherrima* can grow well in the presence of inhibitors. This tolerance is higher than that of many oleaginous yeasts, the majority of which cannot grow at all in the presence of 1 g L^{-1} furfural,[110] whereas we show that the growth of *M. pulcherrima* is reduced by only 50% at a fivefold increase in furfural concentration. *M. pulcherrima* likely has some of the mechanisms adopted by other yeasts for tolerating inhibitors, such as conversion of furfural to furoic acid,[272] HMF conversion to 2,5-bis-hydroxymethlfuran,[287] reduction of oxidative stress, and incorrect protein folding.[280, 288]

3.8 Dilute acid and liquid hot water hydrolysis of wheat straw

Many types of physico-chemical pretreatments have been evaluated for their effectiveness with lignocelluloses; liquid hot water (LHW), dilute acid hydrolysis and steam explosion have emerged as amongst the most industrially applicable physico-chemical pretreatment methods.[233] Hemicellulose is the most thermo-chemically sensitive fraction and begins to solubilise in water at temperatures over 150 °C, with xylose usually the most easily extracted.[341]

Wheat straw was selected as an example second generation lignocellulose feedstock as it is the second largest lignocellulosic waste feedstock globally, and the largest biomass feedstock in Europe (approximately 132 million tonnes are produced annually).[58] *M. pulcherrima* has a high acid tolerance, grows extremely well on di- and trisaccharides, and some evidence in the literature suggests the production of cellulases. On this basis, it was reasoned that it was plausible to attempt to culture the yeast solely on the hydrolysate produced by the chemical pre-treatment stage, without the further enzymatic step that releases monosaccharides. To test the ability of *M. pulcherrima* to be cultured on wheat straw residue a number of scenarios were developed (Table 3.5). The wheat straw used was found to comprise 41% cellulose, 37% hemicellulose and the remaining 22% lignin and ash. In each of these scenarios the wheat straw was hydrolysed using dilute acid

hydrolysis using a range of organic acids and reaction times in a batch reactor. A number of potentially renewable organic acids were selected, as was the more commonly cited sulfuric acid[58, 342] and water (to simulate LHW conditions).[343] For comparison, sulfuric acid was used to hydrolyse wheat straw, both under the same hydrolysis conditions used for the organic acids, under the autoclave conditions frequently cited in the literature (see 2.1.2),[344] and concentrated at room temperature.

Five pretreatment stages were assessed. A similar yield of solubilised wheat straw (57 - 59%) was obtained for each of the organic acids, and slightly less (52%) using water. As expected, only low levels of sugars were obtained from this pretreatment stage. Acetic acid was the least effective at producing monosaccharides from the solubilised wheat straw and similar levels were obtained with this pretreatment as with water. Citric acid was the most effective acid, with 320 mg L^{-1} of xylose being formed. In all the scenarios the majority of the solubilised wheat straw is present as oligosaccharides. On using the organic acids nearly 30 g L^{-1} were produced in all of the scenarios, similar to using H_2SO_4 as the acid catalyst. The wheat straw solubilised with water contained 26 g L^{-1} of solubilised carbohydrate.

No 5-HMF was observed in any of the scenarios, presumably due to the lack of glucose produced in the pretreatment though elevated levels of furfural, a break down product of xylose, were observed. Oxalic acid produced the most, (9.90 g L^{-1}), this is consistent with a low level of xylose but with a reduced solubilised carbohydrate content. Only low levels of acetic acid were observed, suggesting that the furfural was not rapidly breaking down under the conditions employed. The wheat straw pretreated with water produced lower levels of the inhibitors than any of the acid hydrolysed solutions.

M. pulcherrima was then cultured on the wheat straw hydrolysates over seven days (Figure 3.14). The growth was compared to the growth of the control on YMM with 30 g L^{-1} of glucose. *M. pulcherrima* grew poorly on the wheat straw hydrolysate pretreated with organic acids. The starting pH was 3.5, 2.5 and 2.5 for the acetic, citric and oxalic acids respectively. The low growth was seemingly not due to the low starting pH because when raising the pH to 5, similarly poor growth was observed. These acids in high concentrations are probably inhibitory to yeast growth. On the contrary, without pH control, the sulfuric acid treated wheat straw was too acidic for growth, however, on adjusting to pH 5 *M. pulcherrima* grew extremely well. Similarly on treating with water at 121 °C over 30 minutes, enough sugars were released to produce 75% of the biomass when compared to the YMM control media. This suggests that liquid hot water (LHW) pretreatment should be carried forward.

Scenario	S1	S2	S3	S4	S5
Catalyst	acetic	citric	oxalic	sulfuric	water
Concentration (mM)	200	200	200	200	na
Time (min)	120	120	120	120	120
Solubilised yield (% original straw)	59	57	57	57	52
Glu (g L^{-1})	<0.02	0.06	0.05	0.02	<0.02
Xyl (g L^{-1})	0.07	0.32	0.17	0.13	0.03
Ara (g L^{-1})	0.03	0.05	0.08	0.07	0.05
Cel (g L^{-1})	0.00	0.00	0.00	0.00	0.01
Solubilised oligosaccharides (g L^{-1})	29.4	28.4	28.3	28.5	26.0
Furfural (g L^{-1})	2.39	4.86	9.90	7.93	0.03
5-HMF (g L^{-1})	0	0	0	0	0
Acetic acid (g L^{-1})	5.37[a]	0.09	0.08	0.44	0.06
Formic acid (g L^{-1})	1.40	0.74	0.59	0.55	0.66
Aromatics (g L^{-1})	0	0	0	0	0

Figure 3.13. Composition of scenarios for pre-treatment of wheat straw with various catalysts. Temperature ramped to 170 °C and held for the time stated. [a] Acetic acid assumed to derive entirely from the catalyst.

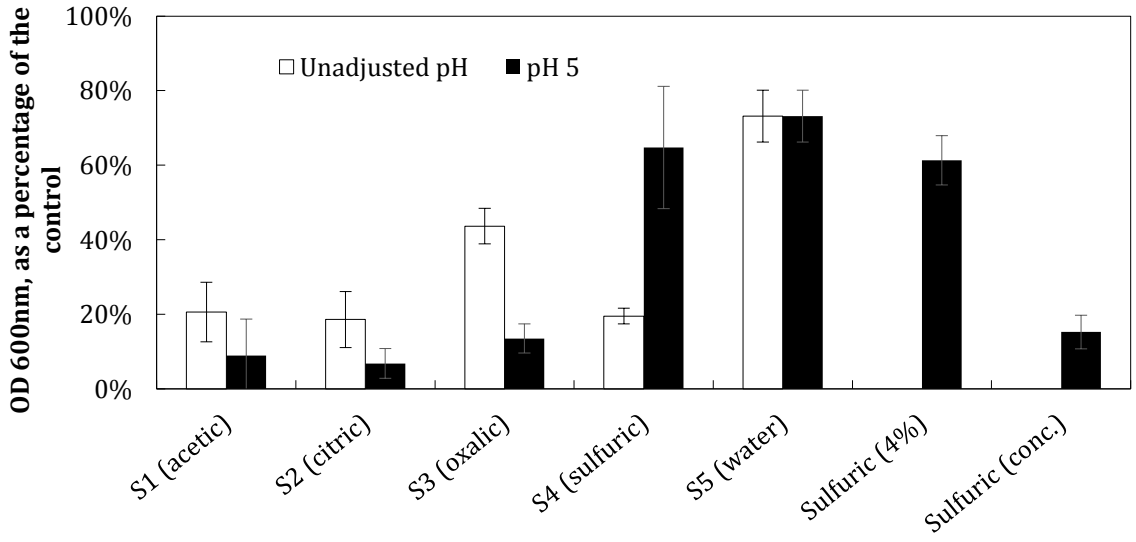


Figure 3.14. Growth by increase in OD of *M. pulcherrima* on a range of scenarios from table 2.5 and also wheat straw hydrolysed by sulfuric acid at 4% and with concentrated (72%). Grown at the pH after hydrolysis and after adjustment to pH 5, over 7 days at 25 °C, 180 rpm.

3.9 Oligosaccharide consumption

In Figure 3.1 and 3.5, it was demonstrated that *M. pulcherrima* catabolises cellobiose, suggesting that it might have the ability to express β-glucosidases in liquid media. Additionally, the high growth of *M. pulcherrima* on the partially depolymerised wheat straw suggests that *M. pulcherrima* has the ability to metabolise the oligosaccharides present in the hydrolysate. To further examine this trait, *M. pulcherrima* was grown on wheat straw hydrolysate treated with sulfuric acid (AHWS, scenario 4), and water treated waste straw (WHWS, scenario 5) by an autoclave hydrolysis process (see 2.1.2). The growth was compared to YMM with 30 g L^{-1} of glucose (control) and two chemically-defined model solutions containing the same monosaccharide concentrations, as well as two ncontaining the same monosaccharide and acid concentrations in AHWS and WHWS but containing none of the oligomers or other hydrolysis products (Figure 3.15). It was thought that the organic acids could act as a carbon source,[159] so the models with and without acids were used.

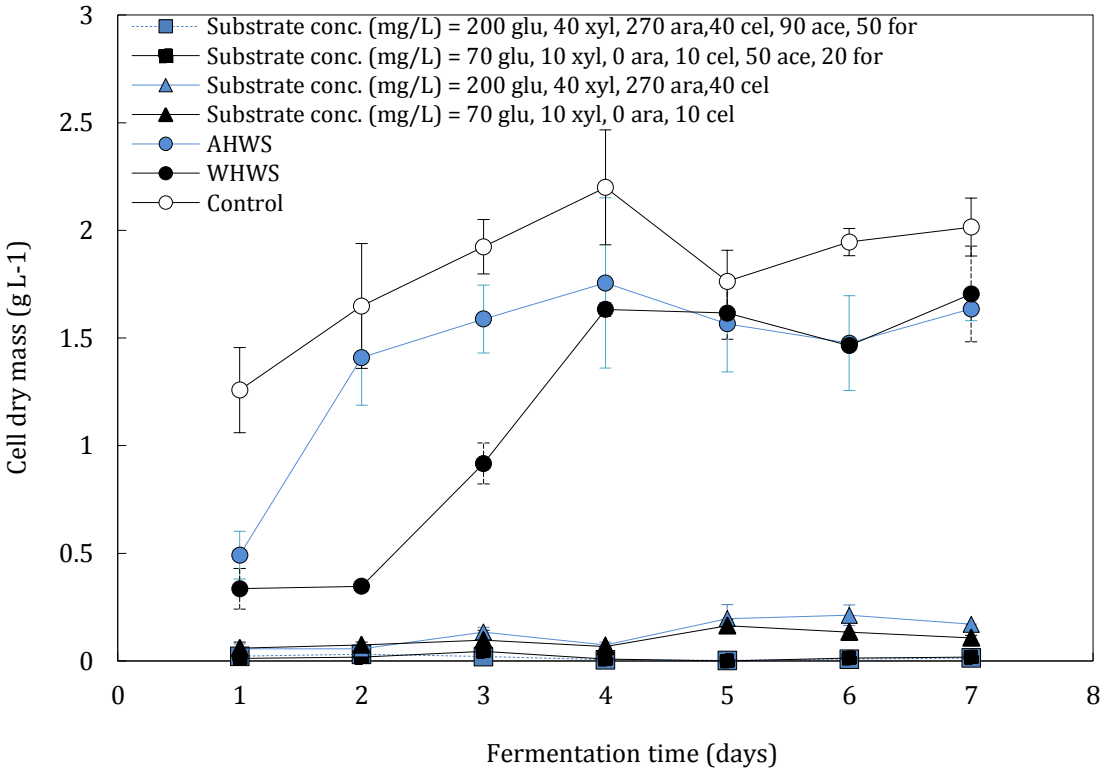


Figure 3.15. Growth of *M. pulcherrima* on AHWS = acid hydrolysed wheat straw (0.1% sulfuric acid , WHWS = water treated wheat straw, both heated for 60 min at 121 °C, control media (YMM with 30 g L^{-1} of glucose) and four model solutions containing the sugars and acids (GLU = glucose, XYL = xylose, ARA = arabinose, CEL = cellobiose ACE = acetic acid, FOR = formic acid) in the concentrations found from AHWS and WHWS.

The yeast entered the stationary phase after two days and a total of 2.4 g L^{-1} of biomass was produced from the control experiment. This is lower than the previous results as glucose is used rather than glycerol and for a shorter time. *M. pulcherrima* grew effectively on the acid hydrolysed water producing about 80% of the biomass of the control. The yeast grew sluggishly at first on the water treated wheat straw though obtained the same level of biomass on reaching the stationary phase as the acid treated straw. In contrast the four model media hardly grew at all, despite having the same sugar (and in two cases the same acid concentrations, but none of the oligosaccharide and polysaccharides that are present in treated wheat straw. This demonstrates that *M. pulcherrima* is capable of utilising the oligomeric saccharides in the supernatant. The fact that *M. pulcherrima* is not utilising acids was confirmed by its inability to grow on neutralised acetic acid containing equimolar concentrations of sugar to the control.

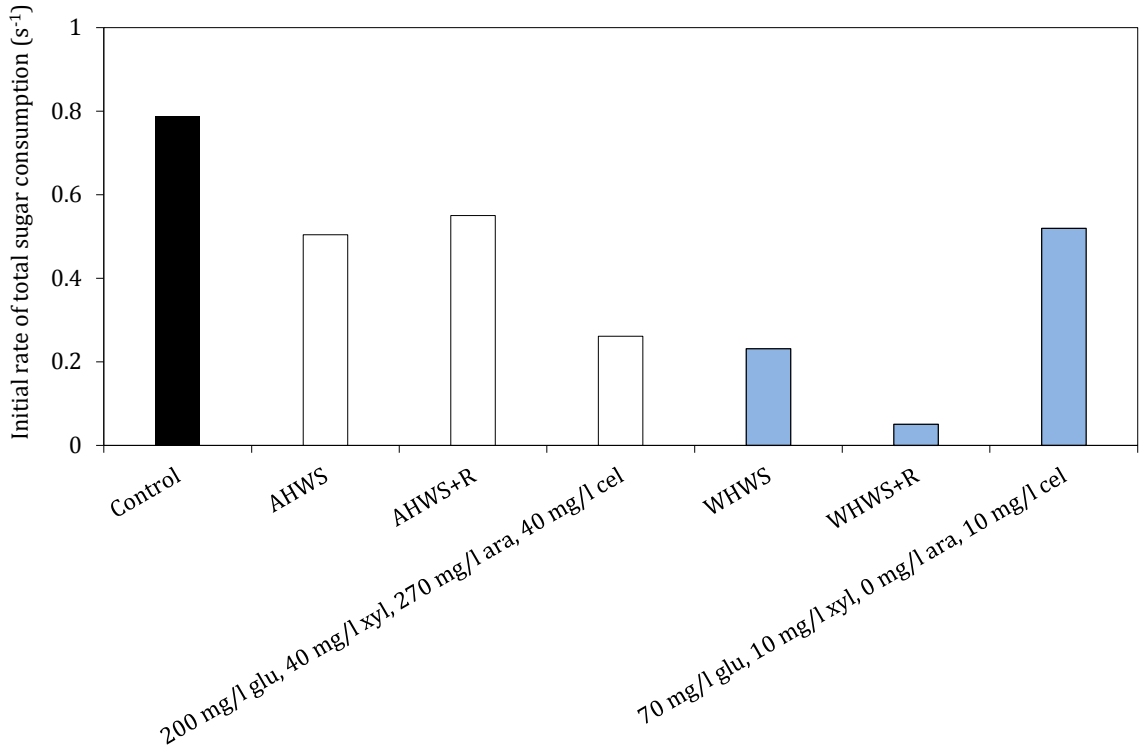


Figure 3.16. Initial rate of total sugar consumption (arabinose, cellobiose, xylose and glucose) during exponential growth phase of *M. pulcherrima* on wheat straw hydrolysates and model wheat straw hydrolysates (as per Figure 3.15) to day four on straw and model sugar solutions measured by HPLC. R = insoluble wheat straw residue

The initial rate of sugar consumption is reasonably high for the acid treated wheat straw, comparable to the rate of initial sugar uptake in the model solutions, and far exceeds the water treated straw (Figure 3.16). This suggests that the rate of monosaccharides

depletion is unaffected by the presence of oligomers or inhibitors. To test the effectiveness of *M. pulcherrima* to catabolise solubilised carbohydrates, *M. pulcherrima* was cultured on the water soluble carboxymethyl cellulose (CMC), and insoluble crystalline cellulose containing variable amounts of glucose (Figure 3.17a) and xylose (Figure 3.17b).

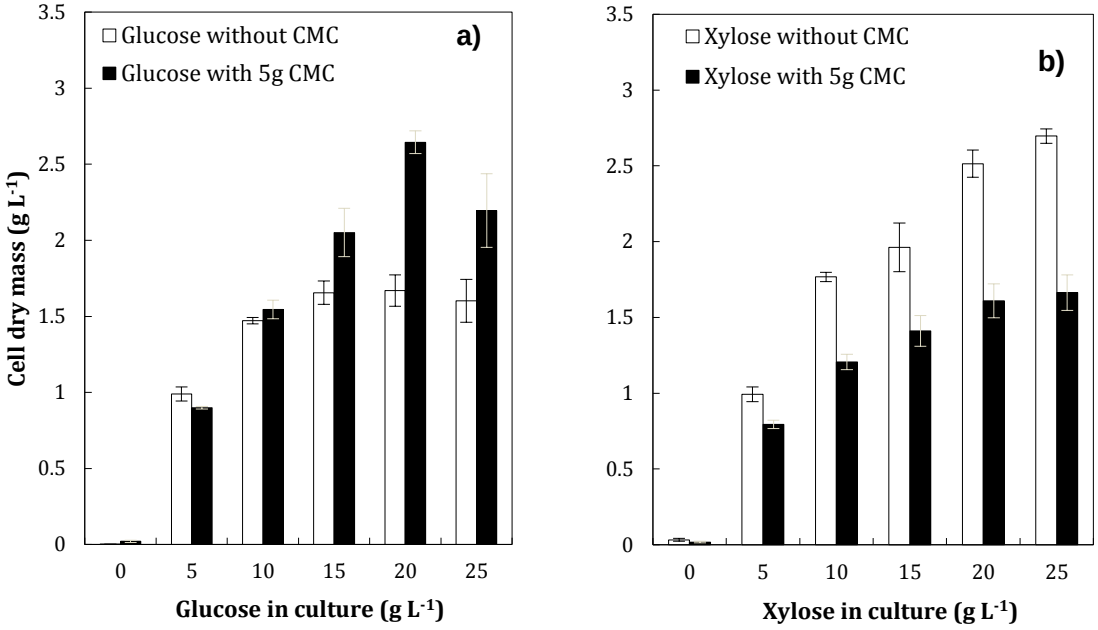


Figure 3.17. Final cell dry mass of *M. pulcherrima* cultures containing the sugars a) glucose (after five days) and b) xylose (after 12 days) with the water soluble polysaccharide carboxymethyl cellulose (CMC).

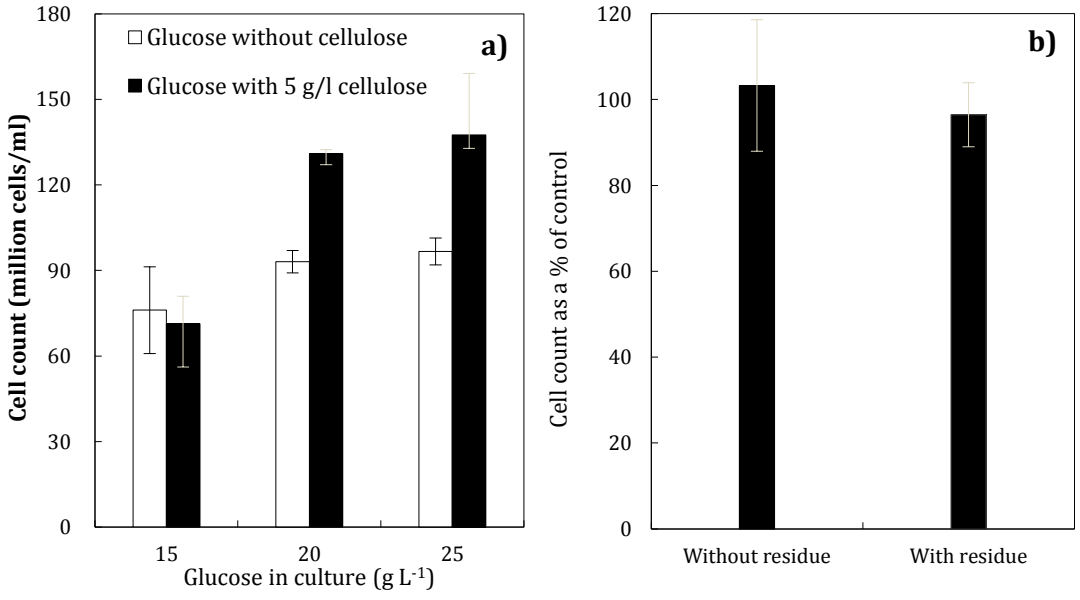


Figure 3.18. Final/relative cell counts of *M. pulcherrima* cultures after fermentation at 25 °C, 180 rpm on a) glucose and insoluble crystalline cellulose for five days, b) AHWS with and without insoluble wheat straw hydrolysate residue.

While the results indicate that *M. pulcherrima* is using the solubilised carbohydrates from the wheat straw, no growth was observed when the sole carbon source was carboxymethyl cellulose (CMC). On addition of 15 g L^{-1} or more of glucose, however, cultures containing the CMC showed a higher overall biomass productivity than those without. As the same effect is not observed with xylose, this suggests that *M. pulcherrima* needs high levels of glucose to produce the necessary enzymes to break down the glycosidic linkages present in the CMC. As the yeast can grow on solubilised oligomers it seems likely that these shorter oligomers can be more readily broken down, producing the necessary energy to break down larger oligomers in the culture. A similar effect was observed for non-soluble cellulose demonstrating that *M. pulcherrima* can use all the available polysaccharides present in the treated lignocellulose. However, the inclusion of genuine non-soluble cellulose in the lignocellulose hydrolysate did not improve the cell count compared to hydrolysate without the residue (Figure 3.18b).

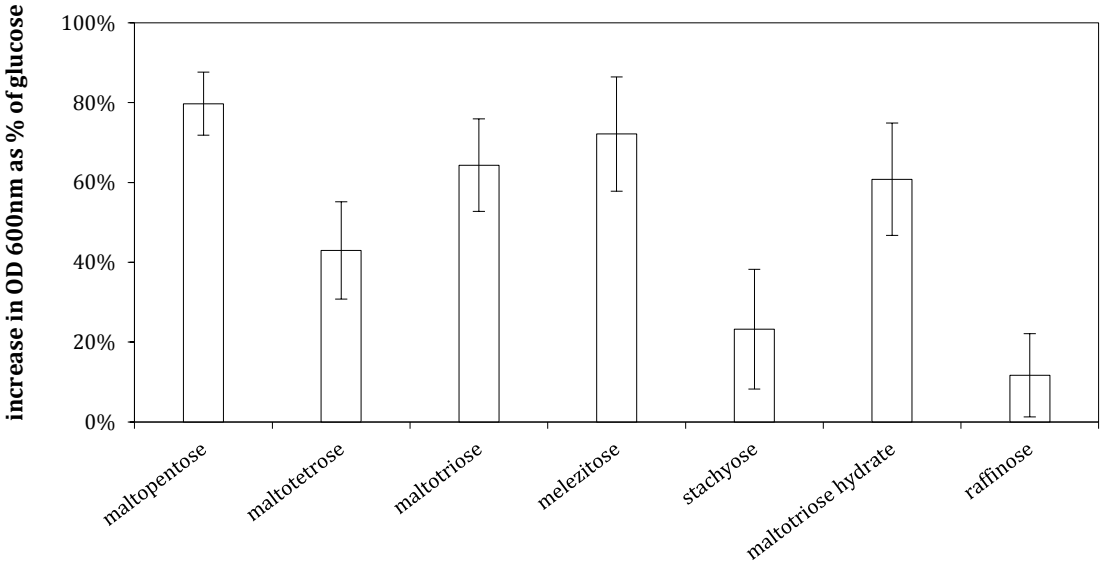


Figure 3.19. Growth of *M. pulcherrima* on individual oligosaccharides at 30 g L^{-1} for 7 days at 25 °C, 180 rpm compared to glucose at 30 g L^{-1} in a 96-well microplate.

This may be that the maximum rate of oligosaccharide hydrolysis is too low to attain any more cell reproduction when non-soluble oligosaccharides are present as well as the soluble oligosaccharides. The β-glucosidase activity is also known to be reduced by low pH and *M. pulcherrima* was shown to reduce the pH of its surroundings (Figure 3.8).

While it seems clear that there is some oligosaccharide catabolism, a large range of oligosaccharides are present. *M. pulcherrima* was therefore cultured on individual oligosaccharides present at 30 $^{L-1}$ and the growth compared to glucose at the same

concentration, with no additional nutrients (Figure 3.19). The growth of *M. pulcherrima* on these oligosaccharides with the only glucose source being from the inoculum (2.5 %vol YPD) was over half that of its growth on glucose in the case of maltopentose, maltotriose, melizitose and maltotriose hydrate. This suggests that *M. pulcherrima* is capable of catabolising these oligosaccharides in liquid media.

3.10 Conclusions

M. pulcherrima was demonstrated to be a highly suitable organism for industrial biotechnology. The yeast could be cultured on two waste resources – glycerol and wheat straw - and made to produce lipid efficiently under low cost, non-sterile conditions. By preventing sporulation through a combination of nitrogen starvation and low temperature, *M. pulcherrima* was made to behave oleaginously, yielding a maximum of 47% lipid in a minimal medium that did not contain yeast extract. *M. pulcherrima* was found to be capable of growing outdoors on glycerol without needing extensive measures to maintain sterility, as such, the yeast has enormous potential as a replacement for palm oil production.

M. pulcherrima was also evaluated for its ability to be cultured on partially hydrolysed wheat straw, avoiding the need for a costly enzymatic depolymerisation process. *M. pulcherrima* was shown to be tolerant to a range of common inhibitors including 5-HMF and furfural (10 mM) and organic acids (up to 60 mM). Wheat straw was partially depolymerised in water at 121 °C over 30 minutes, yielding 0.1 g L^{-1} total monosaccharides, 26 g L^{-1} of solubilised carbohydrates and a range of inhibitors (0.7 g L^{-1}). The *M. pulcherrima* grew well on this mixture yielding 80% of the biomass compared to the control (grown using 30 g L^{-1} glucose). Liquid hot water pretreatment was shown to be the most promising pre-treatment technique, as it does not require additional chemicals while affording reasonable growth. To this end, optimisation of the hydrolysis and culture conditions was undertaken.

Metschnikowia pulcherrima compares favourably with other yeasts that are capable of assimilating oligosaccharides from lignocellulose, as well as the degrading crystalline cellulose. For example, Mangunwardoyo *et al.*[345] reported on a *Trichosporon sporotrichoides* strain which could assimilate cellobiose, carboxymethyl cellulose and microcrystalline cellulose. The oleaginous yeast *Cryptococcus curvatus* was also reported to utilise oligosaccharides, although this was not explicitly demonstrated on authentic lignocellulose.[251] *C. curvatus* is thought to digest the oligosaccharides intracellularly (endogenously). Growth on lignocellulose was also reported for the ascomycete *Coniochaeta ligniaria*, a microorganism that produces cellulases, xylanases and two lignin peroxidases, but without any noticeable laccase activity.[346] Finally, the growth rate and sugar conversion of *M. pulcherrima* matches or in most cases exceeds a range of fungi from the genera *Trichoderma* spp, *Aspergillus* spp, *Fusarium* spp and *Rhizopus* spp, *Piromyces*, *Neocallimastix*, *Orpinomyces* and the thermophilic fungi, *Talaromyces emersonii* and *Thermoascus aurantiacus*, previously investigated for their potential in CBP.[347]

4 Developing lipid production by *M. pulcherrima* on liquid hot water pretreated wheat straw

4.1 Preamble

In the previous chapter, *M. pulcherrima* was shown to be capable of growing on lignocellulose hydrolysate under non-sterile conditions. However, the low volumes of hydrolysate generated by the hydrolysis process in a Parr bomb reactor meant that the growth of yeast was relatively low and the total mass of yeast cells were too small for the lipid content to be gravimetrically determined. In this chapter, a larger scale process was used for the cultivation of *M. pulcherrima* in a temperature and pH controlled 2-L bioreactor, to assess further the applicability of using *M. pulcherrima* as a source of a palm oil substitute.

Liquid hot water (LHW) pretreatment was found to yield as much biomass as the sulfuric acid catalysed treatment so was selected as the hydrolysis method. LHW is a comparatively low temperature hydrothermal pretreatment method that applies above atmospheric pressures to maintain water in a liquid state at high temperatures (160–240 °C) resulting in useful alterations to the structure of the biomass. Catalysts or additional

chemicals are not required and residence times are relatively short (seconds to hours). LHW pretreatment solubilises the majority of hemicellulose allowing access to the cellulose.[348] LHW can convert up to 100% of the hemicellulose, 4-22% of cellulose and 35-65% lignin and thereby greatly enhances the enzymatic digestibility of pretreated material.[327, 349,34] Furthermore, LHW pretreatment is not corrosive and is therefore compatible with low-cost reactors.[233]

Batch processes are typically less economically viable in terms of capital and running costs due to needing more vessels and more down time than continuous processes.[238, 295,192] This has also been demonstrated for the LHW hydrolysis.[234] In these processes, the greater efficiency of flow systems arises from the automatic, consistent continuous operation as well as the possibility of heat recovery. A flow system would thus be desirable in a putative industrial process. Laboratory scale flow systems have been used to hydrolyse rye straw using temperatures of 180, 200 and 220 °C in a packed bed reactor using water (4 mL min^{-1}, 50 Bar). This solubilised between 25%, 43% and 52% of the straw, respectively, yielding a xylose-rich (ca. 40%) liquid fraction, which also contained around 10% lignin and 4% glucose.[350]

In this chapter, *M. pulcherrima* was cultured using a consolidated bioprocessing methodology, where the production of enzymes, saccharification and fermentation by one microorganism in a single vessel was investigated. This reduces the putative capital and running costs substantially while removing the energy and carbon emission impact due to enzyme production.[243, 244] Currently, only one oleaginous yeast has been reported to be cultured in this manner. *C. curvatus* has been demonstrated to produce some oliosaccharide degrading enzymes when cultured on lignocellulose hydrolysate, which produced 15.1 g L^{-1} lipid from corn stover with the addition of cellulase and xylanase, but no β-glucosidase.[157] *C. curvatus* has reasonable inhibitor tolerance, though lower than *M. pulcherrima* , with its growth being reduced by 78% by 1 g L^{-1} furfural.

A batch and a flow LHW process were used to depolymerise wheat straw and compared to conventional heating, *M. pulcherrima* was cultured on the subsequent hydrolysate and the cell dry mass and lipid yield examined. Improvements to the lipid yield were then attempted by finding the optimum parameters for the fermentation conditions using a response surface methodology.

4.2 Liquid hot water hydrolysis - batch process

The LHW hydrolysis process developed in the previous chapter was scaled up from 20 ml to 800 ml at the same water : straw ratio by adapting a previously published method (see section 2.1.2).[137] The solubilisation of the straw was lower than the 20 ml scale at 4% rather than 52% The composition of the hydrolysate consisted mostly of glucose, cellobiose and acetic acid (Figure 4.1), however all components were very dilute.

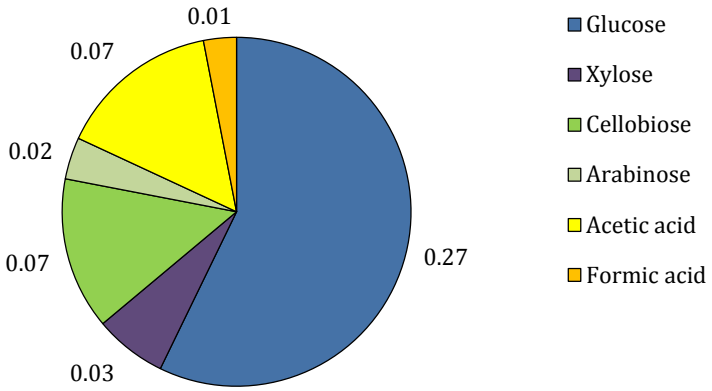


Figure 4.1. Composition of the liquid fraction from LHW pretreated wheat straw (121 °C, 1 hr, 1.034 bar, 800 ml scale batch process), determined by HPLC, excluding water and oligosaccharides. Values given in g L^{-1}.

Several of these hydrolysates were combined and used in a 2 L bioreactor inoculated with *M. pulcherrima* at 25 °C over 3 days at an agitation rate of 250 rpm, an air flow of 2 L min^{-1}, followed by 5 days at an agitation rate of 100 rpm and an air flow of 0.2 L min^{-1}. The temperature, pH, air-flow rate and agitation rate were all automatically controlled. The dissolved oxygen (DO), cell dry mass (by optical density at 600 nm) and hydrolysate composition were measured over eight days. A sample of cells was taken at day 4 and day 8 for lipid content determination (Figure 4.2). The dissolved oxygen content and pH did not change throughout the experiment (see section 2.3.1). The conditions were chosen as preliminary work using glucose had shown them to yield the greatest amount of lipid.

Growth of *M. pulcherrima* reached the stationary phase after three days, with a maximum cell dry mass of 2 g L^{-1}. This was impressive, given that the total mass of sugar and acids measurable by HPLC was 0.47 g L^{-1}, and is 88% of the total amount of straw solubilised (2.5 g L^{-1}). This is further evidence of *M. pulcherrima* being able to metabolise oligosaccharides produced from the hydrolysis of wheat straw, and the ability to do this under controlled conditions.

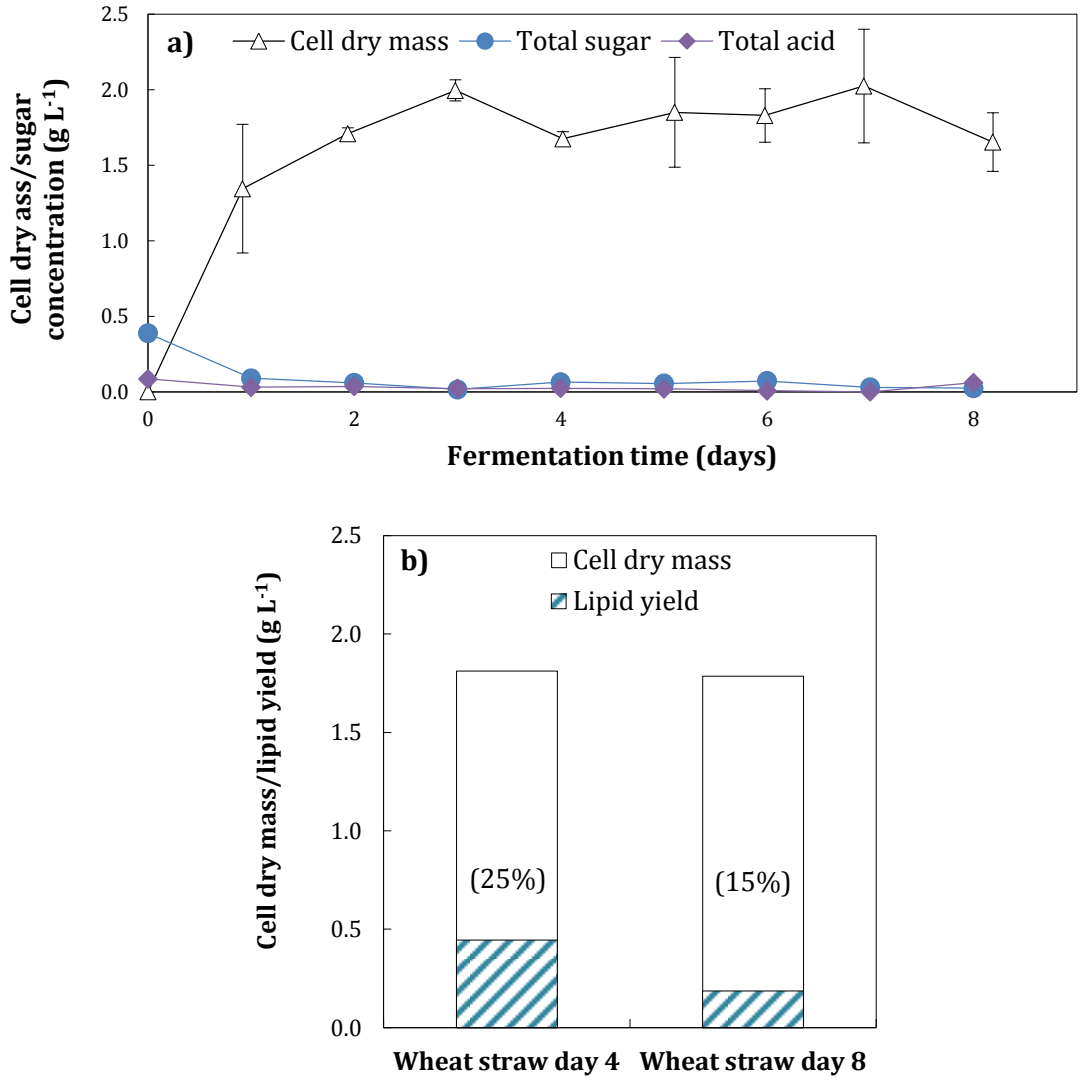


Figure 4.2. a) Cell dry mass (two replicates), total sugar (glucose, xylose, arabinose and cellobiose) concentration, and total acid (acetic, formic and levulinic acids) concentration over fermentation and b) Cell dry mass, lipid yield and (lipid content, %) of *M. pulcherrima* after four and eight days cultivation in non-sterile conditions. Medium was batch LHW pretreated wheat straw in 2 L bioreactors at 25 °C over three days at an agitation rate of 250 rpm, air-flow 2 L min^{-1}, followed by and five days at agitation rate of 100 rpm, air flow 0.2 L min^{-1}.

Monosaccharide concentration decreased most rapidly during the first day's growth, in line with the most rapid growth phase of *M. pulcherrima*, and reached near undetectable levels from day three onwards. The concentration of acids (in the form of conjugate bases acetate and formate) also decreased suggesting that *M. pulcherrima* is either metabolising them or converting them into other compounds.

It was established that *M. pulcherrima* could produce lipid when cultivated on wheat straw hydrolysate. Lipid content was higher at four days than eight (25% vs. 10%), which was

greater than that obtained at these fermentation times by culturing *M. pulcherrima* on glucose under the same conditions (15% after four days, 13% after eight days and reaching 25% lipid only after twelve days). This may be because the sugar is exhausted sooner, so the lipid-rich pulcherrima stage occurs earlier. The lipid yield after four days (0.44 g L^{-1}) is similar to that of *Y. lipolytica* on wheat straw, after detoxification[137] which is at the lower end of the results achieved by oleaginous yeast on lignocellulose. The greatest lipid yield achieved by an oleaginous yeast on wheat straw hydrolysate was by *Rhodotorula mucilaginosa* (5.9 g L^{-1}), and on any lignocellulose hydrolysate was *Cryptococcus humicola* (15.47 g L^{-1}) on corn stover,[125, 309] though all of these hydrolysates contained higher levels of monosaccharides due to the addition of enzymes.

4.3 Liquid hot water hydrolysis – flow process

In order to improve the lipid yield, a higher temperature flow process was used in which liquid hot water at 150 °C, 170 °C and 190 °C was passed over a packed bed reactor charged with wheat straw (see 2.3.2).[350] The higher temperatures are thought to enable softening of the crystalline structure of the cellulose, increasing the yield. This method was chosen for its technical simplicity and non-hazardous nature and was conducted in collaboration with partners at the Hamburg University of Technology. The sugar and inhibitor contents of the resulting Liquid fractions were determined by HPLC, prior to cultivation of *M. pulcherrima* (Figure 4.3)

The samples hydrolysed at 150 °C and 170 °C were very similar in composition, with the wheat straw hydrolysed under the more intense process having slightly higher concentrations of acids, furfurals and sugar. In both hydrolysates, acetic acid was the most significant component, indicating that the hemicellulose is being dehydrated under these conditions. There does not appear to be any selectivity towards glucose liberation.

The sample hydrolysed at 190 °C had a very different composition, with far higher concentrations of all components (except HMF) and 73% of the quantifiable hydrolysis components consisting of glucose (33.47 g L^{-1}). The higher the temperature of the water, the higher its ionic product and thus its capacity to hydrolyse lignocellulose increases.[351] As cellulose does not significantly decompose until 220 °C, the increase in glucose concentration and selectivity is likely to come from the hemicellulose being turned into oligomers then being hydrolysed into glucose.

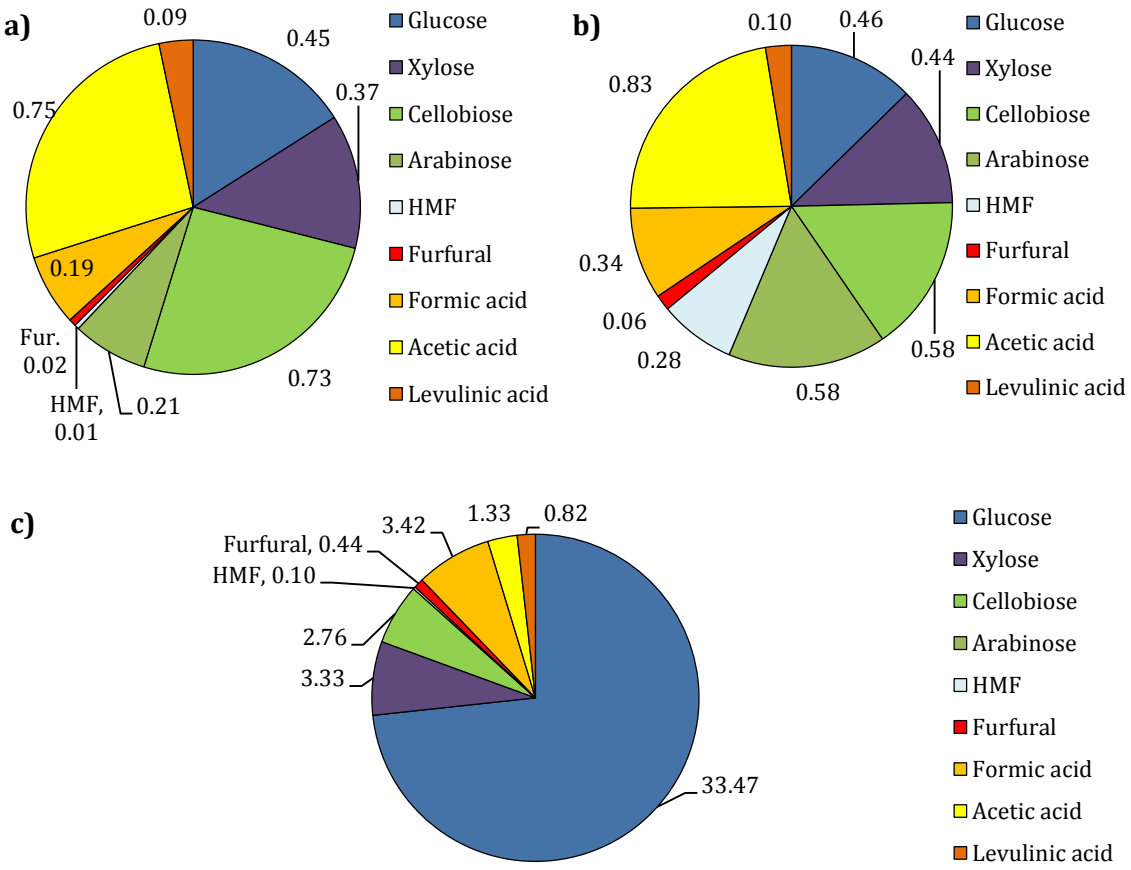


Figure 4.3. Composition of the liquid fraction of wheat straw hydrolysed by LHW in a flow system at a) 150, b) 170 and c) 190 °C, determined by HPLC, excluding water and oligosaccharides. Values in g L^{-1}. Fur = furfural.

M. pulcherrima was then cultured on each of the hydrolysates. Cell dry mass (Figure 4.4) was correlated with hydrolysis temperature, despite the similar compositions of the 150 °C and 170 °C hydrolysates. Cells cultivated in the 190 °C hydrolysate grew better than the YMM control, probably because the total monosaccharide concentration was higher (39 g L^{-1} vs. 30 g L^{-1}), although less well than the control that was held for a longer time (see previous chapter).

Sugar and inhibitor content greatly decreased over the fermentation, as it did in the batch LHW pretreated wheat straw fermentation, utilising 98% (38 g L^{-1}) of the available sugar in the case of the 190 °C hydrolysate (Figure 4.5). Given that this sugar consumption yielded a cell dry mass of 4.7 g L^{-1}, evidently the majority of the sugar was used for respiration and lost as CO_2 rather than increasing the cell dry mass. *M. pulcherrima* may be able to convert furfurals to furfuryl alcohol.[292]

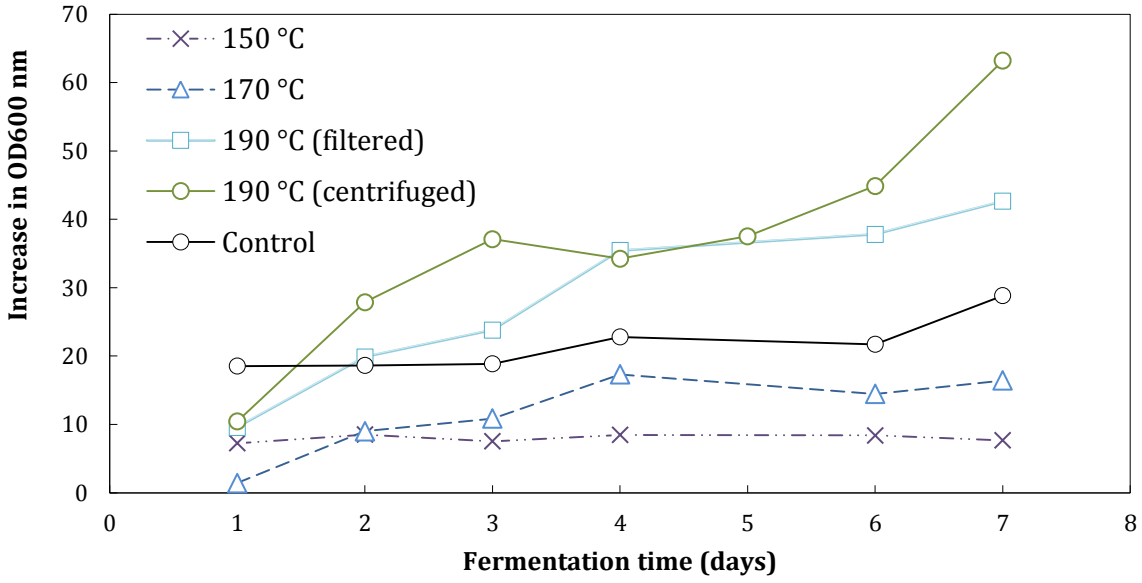


Figure 4.4. Growth of *M. pulcherrima* over seven days at 180 rpm 25 °C, on wheat straw hydrolysed in a flow system at 150 °C, 170 °C and 190 °C. For all samples, solid and liquid fractions were separated by filtration, except for 190 °C, in which a repeated experiment had further solid removed by centrifugation (4000 rpm, 10 min). The control was cultured in YMM under identical conditions.

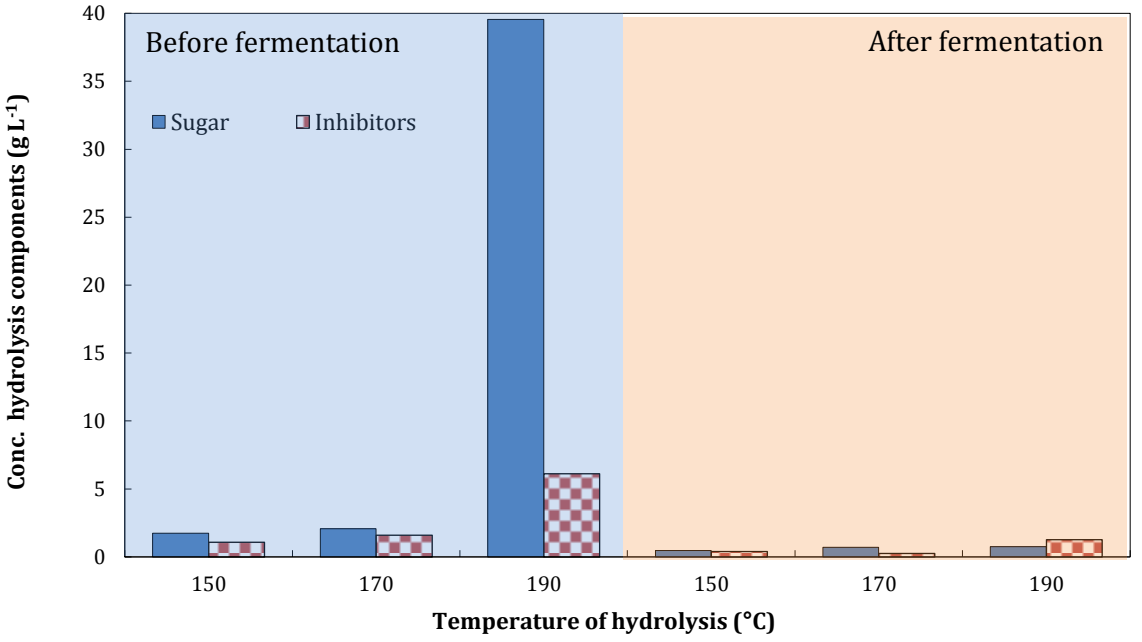


Figure 4.5. Change in hydrolysate composition before fermentation (left) and after fermentation (right). Sugars are glucose, xylose, arabinose, cellobiose, and inhibitors are acetic, formic and levulinic acids as well as furfural and HMF.

The C:N ratio for the 150 °C and 170 °C hydrolysate samples was 16:1, and for the 190 °C sample was 33:1, although not all the carbon or nitrogen was necessarily available to *M.*

pulcherrima. This is a lower ratio than in the nitrogen-limited control medium (58:1), indicating that a significant amount of nitrogen comes from the straw. If accessible to *M. pulcherrima*, this nitrogen source would be beneficial if seeking to increase the cell dry mass. To estimate how much of the nitrogen was utilisable in the 170 °C sample, the concentration of ammonium ions was measured as (0.19 g L^{-1}), which is around half of that of the control medium (0.39 g L^{-1}).

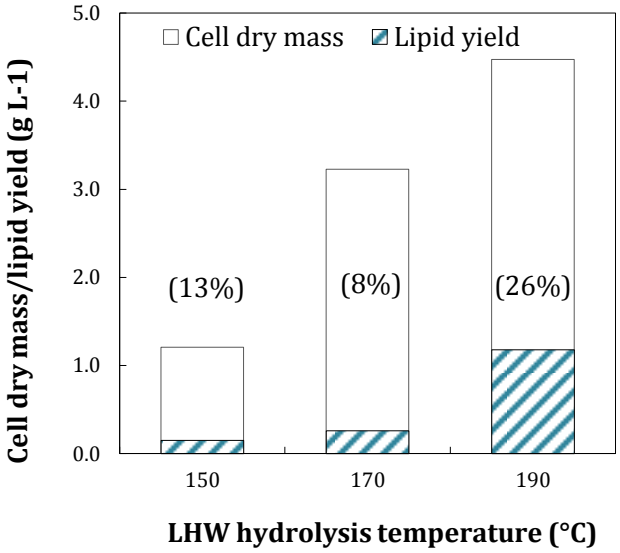


Figure 4.6. Cell dry mass (white bars), lipid yield (striped bars) and (lipid content %) of *M. pulcherrima* cultured on LHW hydrolysed wheat straw at the stated temperatures for seven days.

Regardless of this high nitrogen concentration, the lipid content of the 190 °C sample was in the oleaginous range (26%) and the lipid yield (1.17 g L^{-1}) was improved compared to the batch process (Figure 4.6). It could be that some other stress mechanism is encouraging sporulation but preventing it from occurring, such as the presence of inhibitors or phosphate limitation.[352] Inhibitor tolerant yeasts are known to limit their rate of nitrogen metabolism,[288] so although there is nitrogen present in the media, the *M. pulcherrima* cells may be experiencing low nitrogen concentration within the cell due to the presence of inhibitors.

4.4 Improving the lipid yield by factorial design

As the lipid yield was still lower than comparable literature, the culture conditions were varied in order to find the optimal conditions.[125, 309,137] Response surface methodology is widely used for finding correlations between multiple factors simultaneously, creating models for the behaviour of a system and consequently determining the optimum. Recently, response surface methodology has been applied to lipid production by

oleaginous yeasts.[158, 255, 353-355] The methodology involves screening a wide range of variables (factors) and finding correlations between factors and dependent variables (responses). In complex systems such as these, there are often interactions between factors that may also be correlated with the responses. It was assumed that addition of supplemental nutrients as per the control medium would lead to improved lipid yields and that the optimum pH was still 5, so these factors were kept the same for all fermentations. Seven varying factors were then selected:

- **Shaking speed**. Increasing saturation by dissolved oxygen of the fermentation media is thought to increase the lipid content of oleaginous yeast.[125, 180, 190] On an industrial scale, this can be altered by the flow rate of the air input or by the reactor design (e.g. airlift reactors), but on the scale of shake flasks this can be achieved by increasing the shaking speed.[356]
- **Inoculum type.** YMS (30 g L^{-1} yeast extract, 5 g L^{-1} mannitol, 5 g L^{-1} sorbose) is reported to give higher yields of *M. pulcherrima* in shorter times than YPD (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose) reaching 6 g L^{-1} in 13 hours.[357]
- **Inoculum volume.** Increasing the inoculum volume has been reported to improve growth and lipid content of *T. fermentans* and *C. curvatus* in model lignocellulose hydrolysates with inhibitors present, presumably as inoculum media replaces nutrient poor, inhibitor rich hydrolysate.[156, 292] On authentic lignocellulose hydrolysates, the majority of oleaginous yeast studies have used a 10% inoculum volume. [190, 307, 309]
- **Temperature (T).** Although *M. pulcherrima* was shown to produce greater lipid contents at lower temperatures on glycerol and glucose, the lipid yield was not much altered at varying temperatures as the cell dry mass decreases at lower temperatures. Preliminary experiments suggested that an optimum temperature for lipid content could lie at 18 °C rather than 15 °C, but temperature had not been examined on wheat straw hydrolysates.
- **Presence of Tween 80.** Zhao *et al.* included 2 g L^{-1} of the surfactant Tween 80 for an unspecified reason when cultivating *C. curvatus* on corn stover.[157] At such a significant concentration compared to sugars and supplemental nutrients and containing a fatty acid, it was thought that this might have an influence on the growth of the yeast. It could prevent flocculation and so increase the total surface area of the yeast available for oxygen and nutrient absorption, or the oleic acid component could be consumed by the yeast.
- **Concentration of potassium phosphate dibasic (K_2HPO_4, symbol = KPO3).** The amount of phosphate used in yeast media by researchers varies, for example,

Chatzifragkou *et al.*[95] used 7 g L^{-1} of K_2HPO_4 and 2.5 g L^{-1} of sodium phosphate monobasic, whereas Zhao *et al.*[157] used 2.7 g L^{-1} K_2HPO_4 and 2.5 g L^{-1} of sodium phosphate monobasic. Phosphate is an essential nutrient used in ATP, nucleotide and membrane lipid synthesis, and so its concentration could affect cell dry mass and possibly lipid content. The less that can be added, the cheaper the process will be. Phosphate limitation can also be used to induce lipid accumulation, in a similar protocol to nitrogen limitation.[352]

- **Concentration of yeast extract (YE).** Nitrogen limitation is known to induce lipid production, and the correct level must be found for each medium.[133, 142, 173] Yeast extract is a nitrogen source (%N = 10), in addition to providing other nutrients.

The statistical software package MODDE (Umetrics, Sweden) was used to generate the design and calculate the response surfaces. A fractional factorial design was selected in order to find the factors with the strongest impact on the cell dry mass, lipid content and lipid yield with a limited quantity of hydrolysate. Although the hydrolysis process at 190 °C provided the best results, there was an insufficient quantity of this sample, and so the 170 °C sample was used. *M. pulcherrima* was cultured on the wheat straw hydrolysate and the values for cell dry mass, lipid content and lipid yield were determined. Models for each response were then defined by examining the correlation between all factors (and interactions) and the responses. Factors that were insignificant (defined by those whose removal led to the increase in the Q2 value, a measure of the predictive power of the model (maximum value = 1).

In order to examine the accuracy of the models in predicting the responses, the values predicted for each of the scenarios in Table 4.1 are compared to the observed values for these scenarios (Figure 3.7). If the model were perfect, the predicted values and observed values would be identical, so the correlation between these values would be linear and highly significant. The null hypothesis that there is no correlation between the observed and predicted values was analysed by ANOVA (Figure 4.7 and table 4.2).

Shaking (rpm)	Inoculum size (%vol)	Inoculum type	T (°C)	Tween 80 (g L^{-1})	Conc. K_2HPO_4 (g L^{-1})	Conc. YE (g L^{-1})	Dry weight (g L^{-1})	Lipid content (%)	Lipid yield (g L^{-1})
60	2.50	YPD	10.0	2	1	0	2.88	11.05	0.32
60	2.50	YPD	10.0	2	1	0	3.58	6.74	0.24
60	2.50	YPD	10.0	2	1	0	3.06	14.31	0.44
60	2.50	YMS	10.0	0	7	1	3.30	6.58	0.22
60	2.50	YMS	10.0	0	7	1	3.13	4.21	0.13
60	2.50	YMS	10.0	0	7	1	5.48	4.99	0.27
200	20.0	YPD	10.0	0	7	0	6.44	5.18	0.33
200	20.0	YPD	10.0	0	7	0	7.02	10.35	0.73
200	20.0	YPD	10.0	0	7	0	6.00	6.85	0.41
200	20.0	YMS	10.0	2	1	1	4.92	5.96	0.29
200	20.0	YMS	10.0	2	1	1	6.16	9.85	0.61
200	20.0	YMS	10.0	2	1	1	9.06	6.62	0.60
130	11.25	YMS	17.5	0	4	1	6.61	12.64	1.51
130	11.25	YMS	17.5	0	4	1	6.61	6.17	0.41
130	11.25	YMS	17.5	0	4	1	6.35	13.15	0.83
130	11.25	YMS	17.5	0	4	1	6.19	4.68	0.29
130	11.25	YMS	17.5	0	4	1	6.23	9.05	0.56
130	11.25	YMS	17.5	0	4	1	6.71	6.04	0.41
130	11.25	YMS	17.5	0	4	1	5.03	6.16	0.31
130	11.25	YMS	17.5	0	4	1	7.15	5.33	0.38
130	11.25	YMS	17.5	0	4	1	6.74	9.52	0.64
200	2.50	YMS	20.0	2	7	0	4.02	7.68	0.08
200	2.50	YMS	20.0	2	7	0	4.33	11.19	0.48
200	2.50	YMS	20.0	2	7	0	4.00	10.77	0.43
200	2.50	YPD	20.0	0	1	1	2.63	5.03	0.13
200	2.50	YPD	20.0	0	1	1	3.61	23.87	0.86
200	2.50	YPD	20.0	0	1	1	2.54	0.84	0.02
60	20.0	YMS	20.0	0	1	0	2.79	8.78	0.25
60	20.0	YMS	20.0	0	1	0	4.53	8.88	0.40
60	20.0	YMS	20.0	0	1	0	4.34	10.31	0.45
60	20.0	YPD	20.0	2	7	1	5.81	0.66	0.04
60	20.0	YPD	20.0	2	7	1	4.85	10.07	0.49
60	20.0	YPD	20.0	2	7	1	3.08	13.77	0.42

Table 4.1. Factors (left) and responses (right). Factors and levels defined by fractional factorial, resolution III in MODDE. Cultured in triplicate

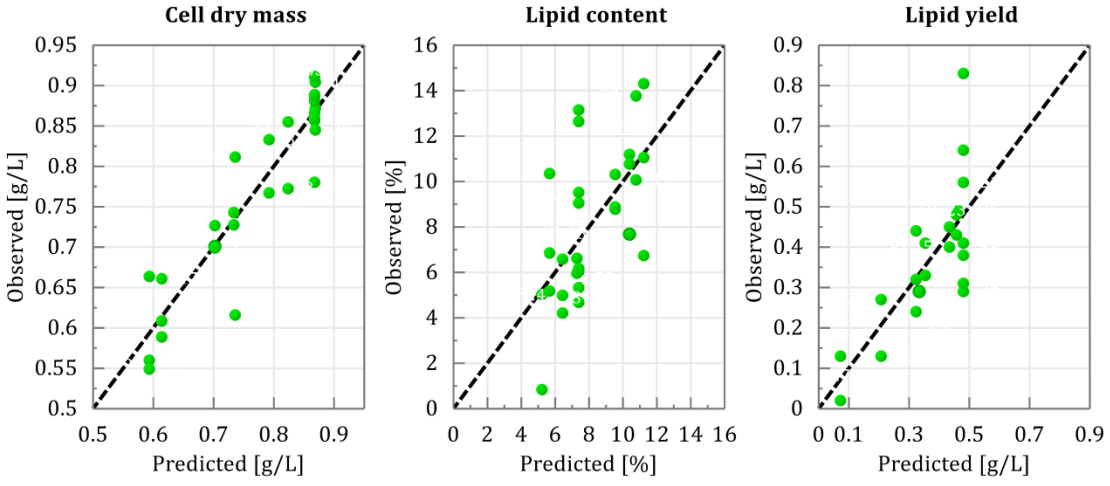


Figure 4.7. Correlations between predicted values and observed values for cell dry mass.

	Degrees of freedom	R^2	F	p
Cell dry mass	20	0.86	15.22	0.000
Lipid content	22	0.38	1.93	0.112
Lipid yield	14	0.56	2.25	0.088

Table 4.2. Results of ANOVA analysis of models.

The models accurately described the experimental data in the case of the cell dry mass (R^2=0.86), and less so for the lipid yield (R^2=0.56) which indicated successful, if weak, correlation between the model's predictions and the observed values. The p values of 0.000, 0.112, and 0.088 for cell dry mass, lipid content and lipid yield respectively indicate the significance of the correlations - less than ideal (95%) in the case of lipid content and yield, yet still high. The reason for the unexplained variance may be due to the imprecision of the lipid content data (see section 2.1.5).

4.4.1 Cell dry mass model

The models may be summarised by the factors (and interactions between factors) which influence the response. The factors affect the response to varying extents, as shown in Figure 4.8. Several of the factors selected did not have a significant effect on the cell dry mass, so do not appear in Figure 4.8.

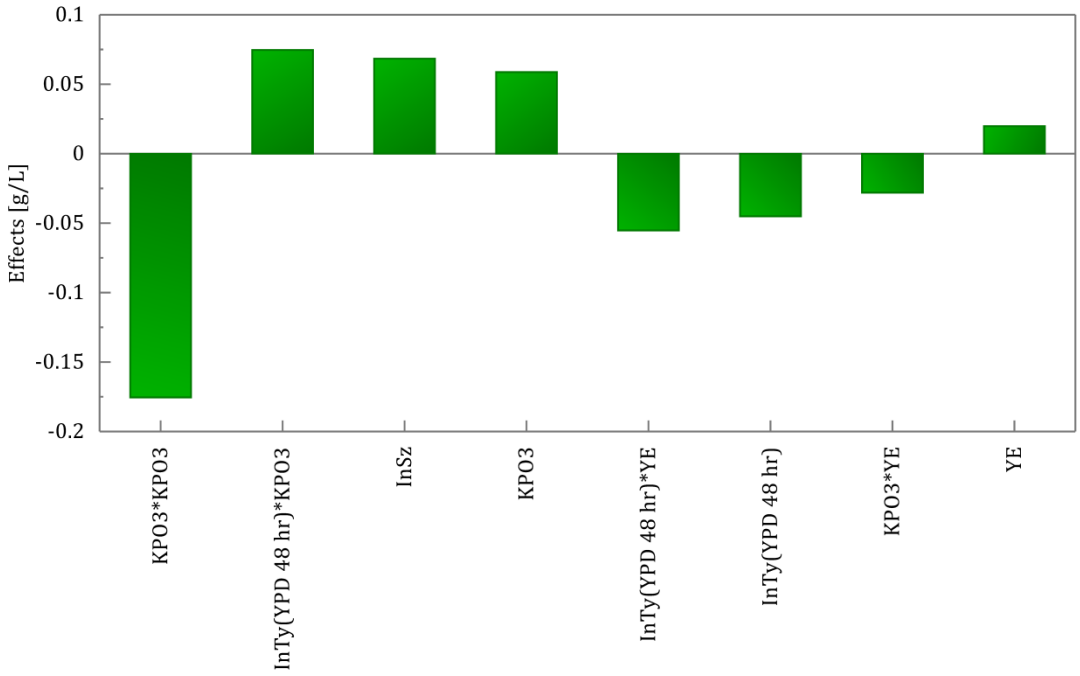
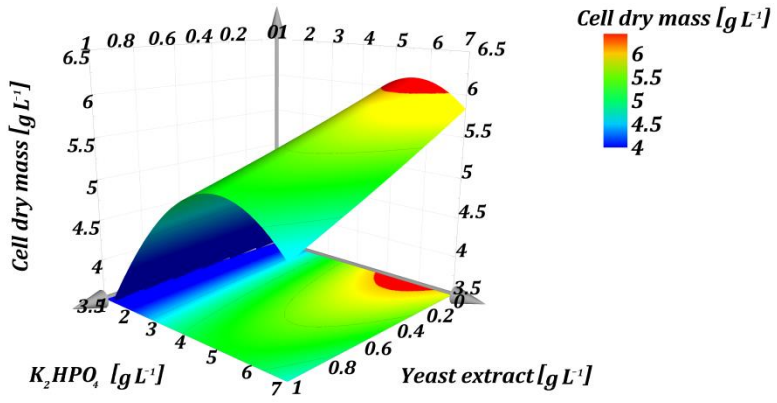


Figure 4.8. Positive and negative effects of factors (and interactions (*) between factors) with cell dry mass of *M. pulcherrima* cultivated for seven days on wheat straw pretreated at 170 °C. InSz = inoculum size, InTy = inoculum type, KPO3 = potassium phosphate, YE = yeast extract.

The lack of effect of temperature over the range studied (10-20 °C) corroborates the findings of the raceway pond culture. If increasing the cell dry mass were the only objective, this could mean that one of the most expensive control systems in a process could be negated. The response surfaces predicted by the models indicate the most important factors and the direction the design space should be moved towards, if unconstrained by techno-economic factors.

a)

YPD (11.5% vol)



b)

YMS (11.5% vol)

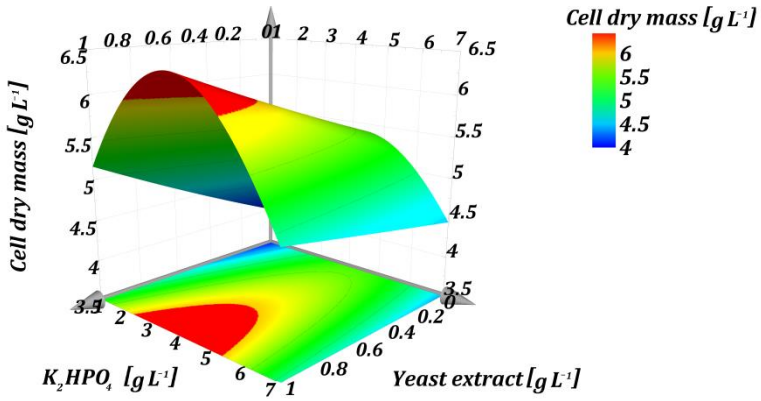


Figure 4.9. Predicted cell dry mass of *M. pulcherrima* cultivated for seven days at any temperature or shaking speed with no additional Tween 80 on wheat straw hydrolysed at 170 °C with varying quantities of additional yeast extract, and potassium phosphate (K_2HPO_4) and inoculated with 11.5% of either a)YPD or b)YMS inoculum (48 hours pre-incubation).

Shaking speed also does not show a strong correlation with cell biomass, indicating that the shake flask system provides adequate oxygen for cell division. The presence of Tween was also unimportant for the growth of *M. pulcherrima* and could be left out of an industrial fermentation. On the other hand, the size and type of inoculum had a strong effect on the cell mass. YMS was far better for cell dry mass than YPD, as can be seen in Figure 4.8 and by comparing a with b for Figure 4.9Figure 4.10, and Figure 4.11.

Interestingly, despite the fact that YMS has three times the concentration of YPD, if YMS inoculum is used the maximum amount of yeast extract (1 g L^{-1}) should be added, but if YPD inoculum is used it is preferable to add no additional yeast extract to the hydrolysate.

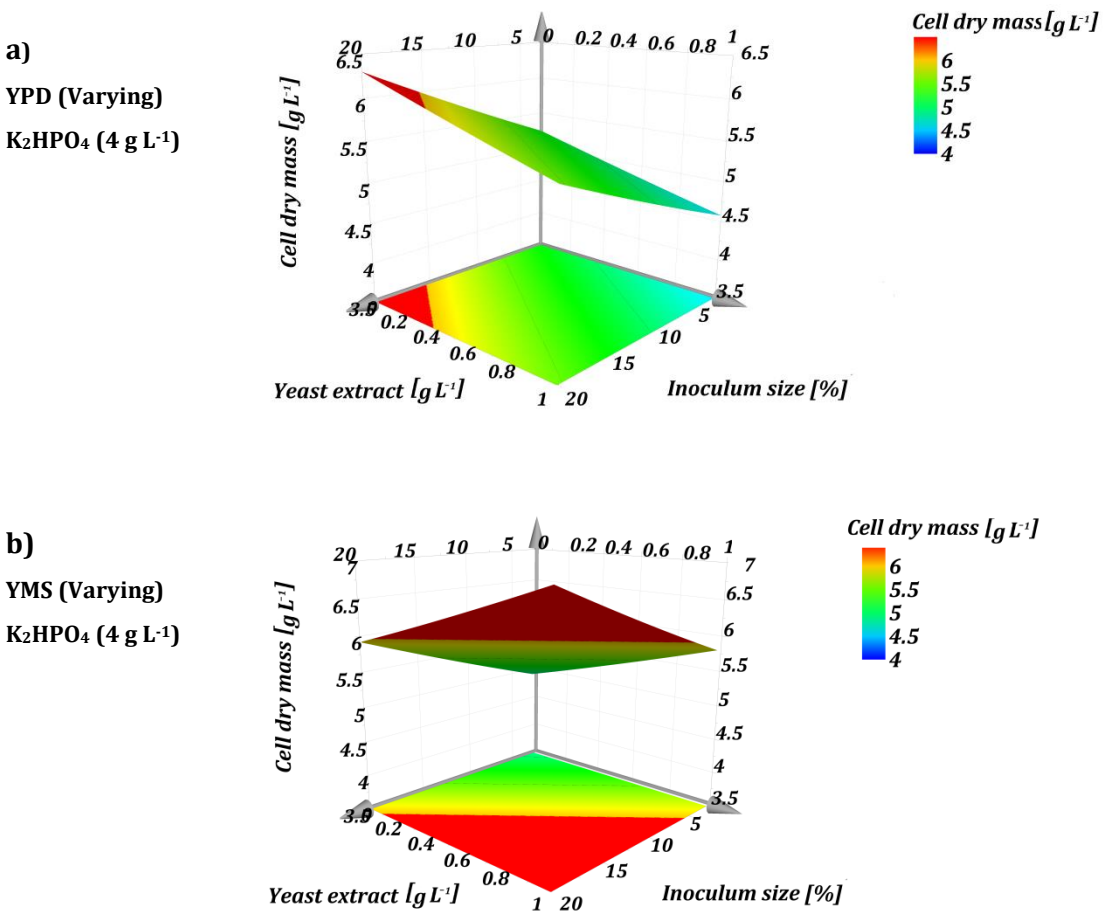


Figure 4.10. Predicted cell dry mass of *M. pulcherrima* cultivated for seven days at any temperature or shaking speed with no additional Tween 80 on wheat straw hydrolysed at 170 °C with 4 g L^{-1} potassium phosphate (K_2HPO_4) varying quantities of additional yeast extract, and inoculated with varying amounts of either a) YPD or b) YMS inoculum (48 hours pre-incubation).

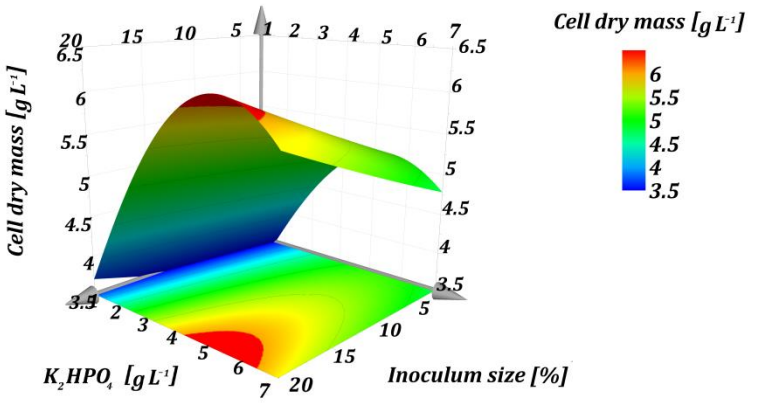
The reason for this effect may be that when *M. pulcherrima* is initially growing in the liquid inoculum it adapts its enzymes to a yeast extract-rich environment, and so has a shorter lag phase during the main culture when more yeast extract is present in the hydrolysate. Consequently, the highest cell dry mass is obtained when both the maximum volume of YMS (20%) and the maximum amount of yeast extract (1 g L^{-1}) are added (Figure 4.10). Such conditions would be costly, so a balance would need to be struck for a suitable industrial process aimed at maximising cell dry mass.

a)

YPD (Varying)

Yeast extract

(0.5 g L^{-1})



b)

YMS (Varying)

Yeast extract

(0.5 g L^{-1})

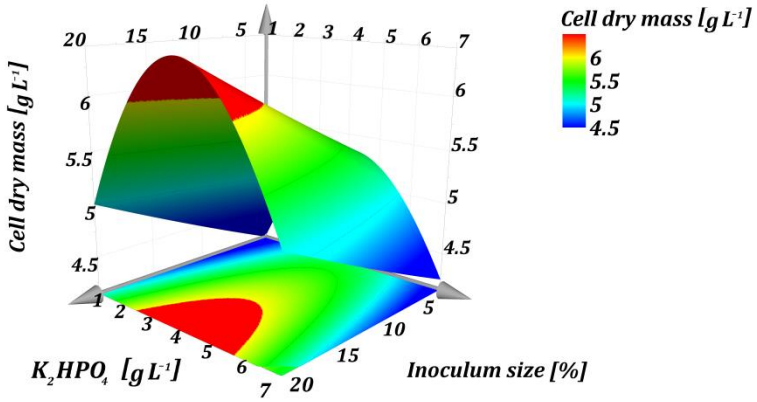


Figure 4.11. Predicted cell dry mass of *M. pulcherrima* cultivated for seven days at any temperature or shaking speed with no additional Tween 80 on wheat straw hydrolysed at 170 °C with 0.5 g L^{-1} of additional yeast extract, and varying potassium phosphate (K_2HPO_4) and inoculated with varying quantities of either a)YPD or b)YMS inoculum (48 hours pre-incubation).

There is an interaction between K_2HPO_4 and itself (Figure 4.8) which has a negative effect on the cell dry mass, meaning that there are minima in the cell dry mass at low and high concentrations of potassium phosphate (Figure 4.9 and Figure 4.11). This could be a case of higher concentrations of phosphate, or potassium, becoming toxic. Interactions between YPD inoculum and both potassium phosphate and yeast extract cause the asymmetric shape of the plots in Figure 4.9a and Figure 4.11a.

4.4.2 Lipid content

An identical process was used to determine the lipid content model and examine its predictions. The effects of the factors that significantly affect the lipid content are shown in Figure 4.12.

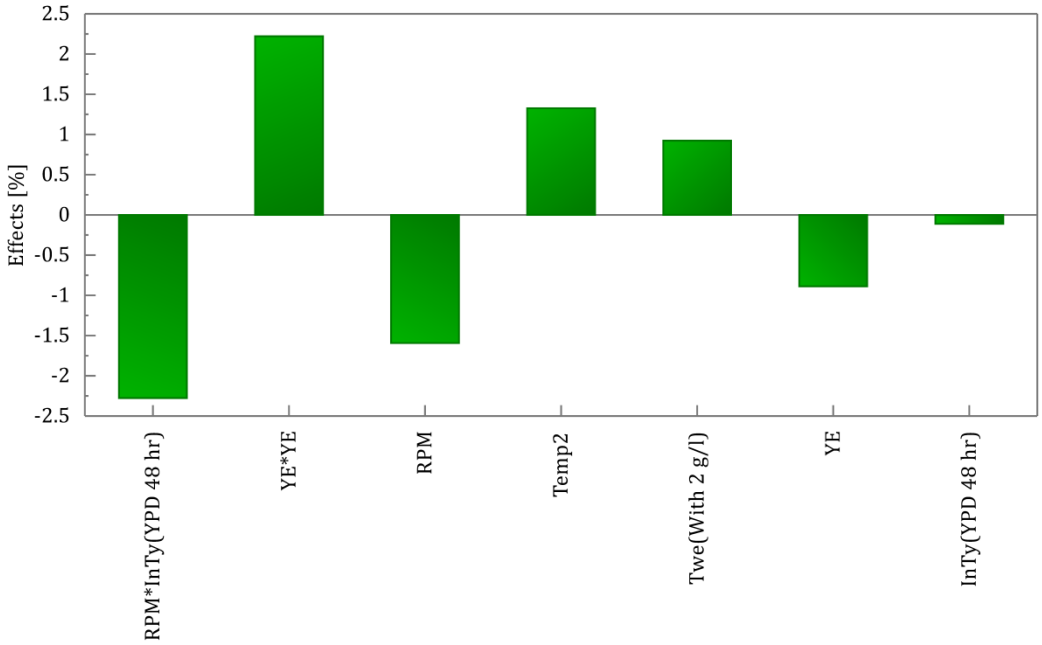


Figure 4.12. Positive and negative effects of factors (and interactions (*) between factors) on lipid content of *M. pulcherrima* cultivated for seven days on wheat straw pretreated at 170 °C. rpm = shaking speed; InTy = inoculum type, YE = yeast extract Temp2 = temperature for days 4-7.

For the lipid content, shaking speed is significant, however it interacts with the inoculum type (Figure 4.12). This results in the lipid content being weakly positively correlated with shaking speed when YMS inoculum is used (as would be expected), and more strongly negatively correlated when YPD is used, contrary to what was predicted (Figure 4.13a and b). YPD generally gave higher lipid contents than YMS under otherwise identical conditions as it contains less nitrogen.

If oxygen concentration in the bulk aqueous phase is directly correlated with lipid content, the mass transfer between the air and the bulk liquid would have to be greater when the shaking speed was low, however this is not the case. Shaking speed is directly correlated with the mass transfer coefficient of oxygen in shake flasks[356] and in industrial fermentations.[358] It therefore appears that in the case of YPD inoculum, oxygen concentration is not correlated with lipid content.

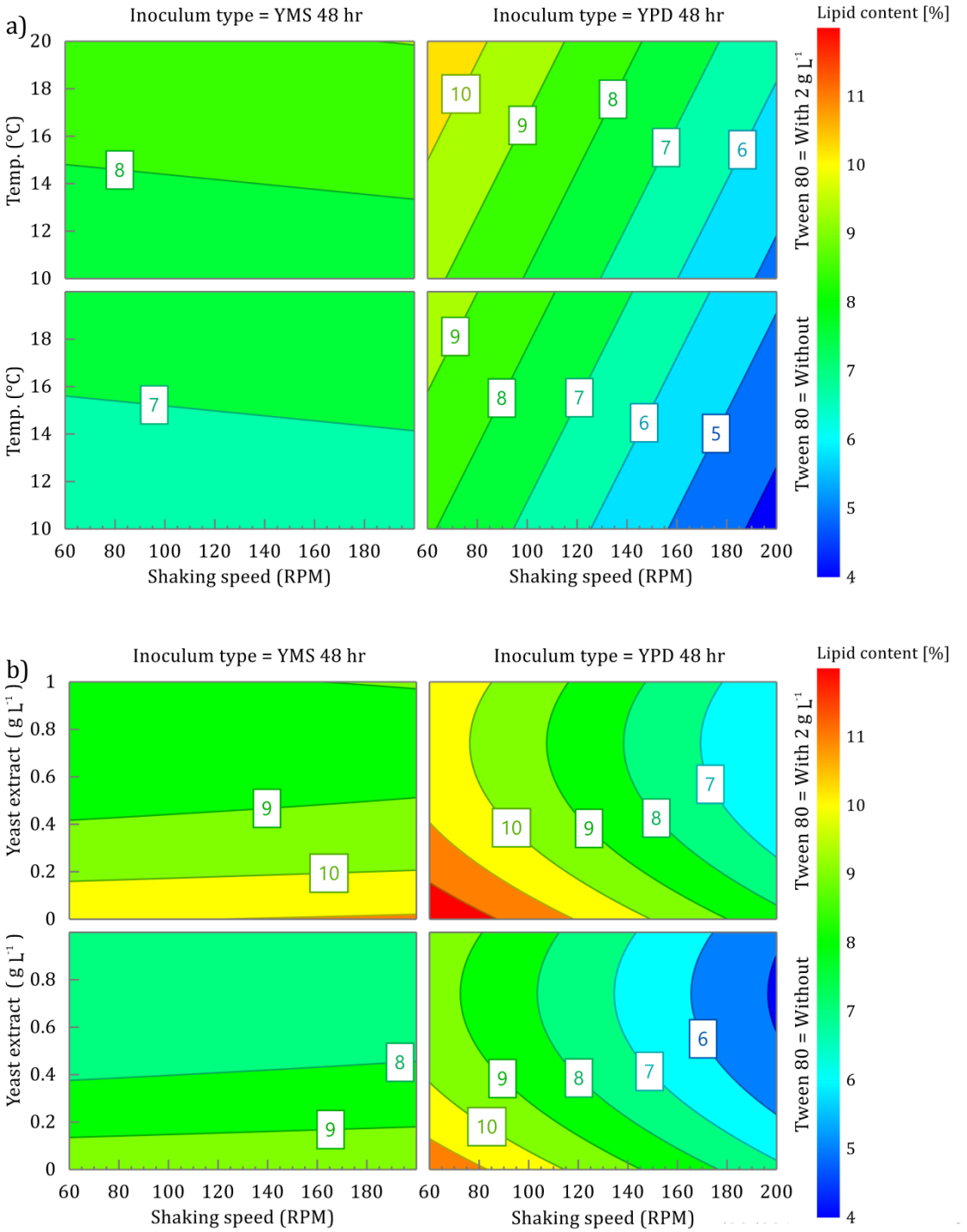


Figure 4.13. Predicted lipid content of *M. pulcherrima* cultivated for seven days at any inoculum size, with any amount of K_2HPO_4 on wheat straw hydrolysed at 170 °C by a LHW flow process with varying quantities of additional yeast extract, and inoculated with varying amounts of either YMS (left) or YPD (right) inoculum with (top) or without (bottom) Tween 80 (2 g L^{-1}) and with varying amounts of a) yeast extract, and b) temperature with shaking speed

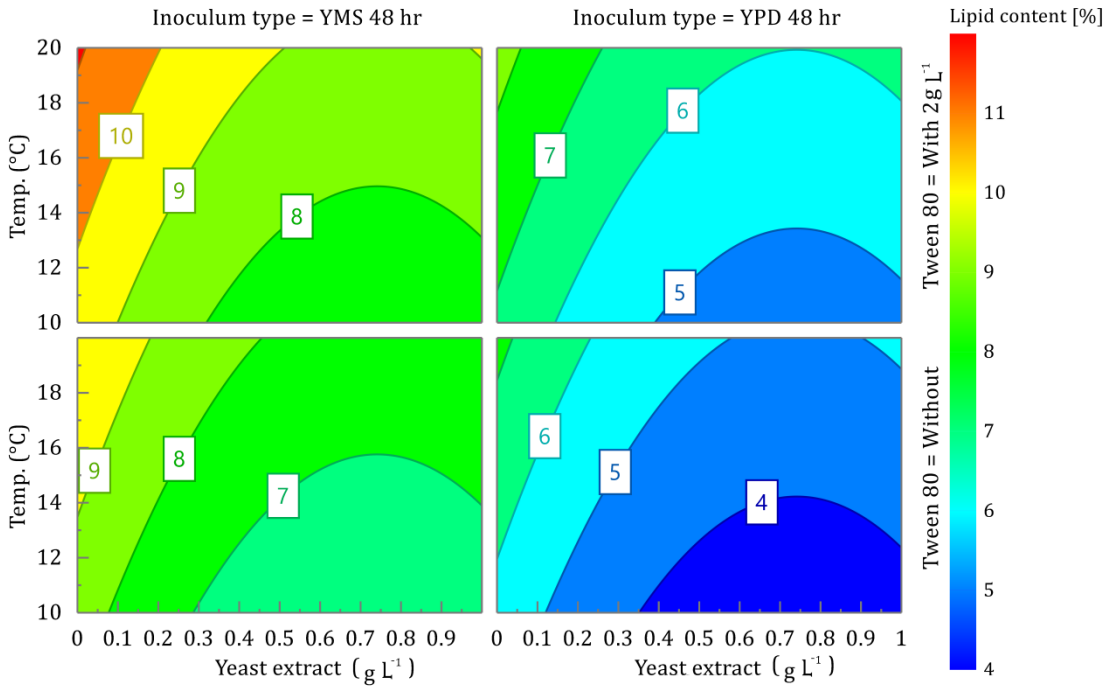


Figure 4.14. Predicted lipid content of *M. pulcherrima* cultivated for seven days on wheat straw hydrolysed at 170 °C by a LHW flow process either inoculated with any amount of YMS (left) or YPD (right) inoculum , with additional K_2HPO_4 with (top) or without (bottom) Tween 80 (2 g L^{-1}) and with varying amounts of yeast extract, temperatures and shaking speeds.

Surprisingly, although inoculum type was important, inoculum size was insignificant. This is unlike the results obtained for *C. curvatus* in model lignocellulose hydrolysates containing individual inhibitors.[156] The reason for the low lipid content compared to YMM may therefore be more to do with the difficulties in catabolising sugar than overcoming inhibitors, or that increasing the inoculum volume is only sufficient to overcome inhibition when individual inhibitors are present. The potassium phosphate content was also unimportant, perhaps indicating that the phosphate content was too high in the wheat straw hydrolysate for phosphate limitation to occur. Lipid content is highest when minimal yeast extract is added, regardless of the medium used for the inoculum. This indicates that there is sufficient accessible nitrogen in the hydrolysate for growth and any additional nitrogen delays, or prevents, nitrogen starvation. There is an interaction between yeast extract and itself that suggests that extending the design space beyond 1 g L^{-1} yeast extract might further increase the lipid content (Figure 4.14), however this is unlikely to improve the economics of the process. Given the inverse correlation between nitrogen concentration and lipid content, the benefits to the lipid content at higher concentrations are likely to come from the non-nitrogenous constituents of the yeast extract. Additional nutrients from a cheaper source could therefore be beneficial. The

presence of Tween 80 increases the lipid content, but only by 1% wt, suggesting that the oleic acid component may be being utilised by the yeast for membrane synthesis. This is unlikely to make up for the costs of adding this component.

Interestingly, higher temperatures give greater lipid contents, in contrast to the growth of *M. pulcherrima* on glycerol (Figure 4.14a and c). Additionally, none of the results for lipid content on this particular hydrolysate are in the oleaginous range although they were for the 190 °C hydrolysate. This suggests that either fewer lipid-rich "pulcherrima" cells form, or sporulation occurs before harvesting the cells, regardless of temperature. Given that the maximum lipid content was obtained after four days in the batch process, the high stress conditions may be inducing sporulation earlier

4.4.3 Lipid yield

The effects of the factors that significantly affect the lipid content are shown in Figure 4.15.

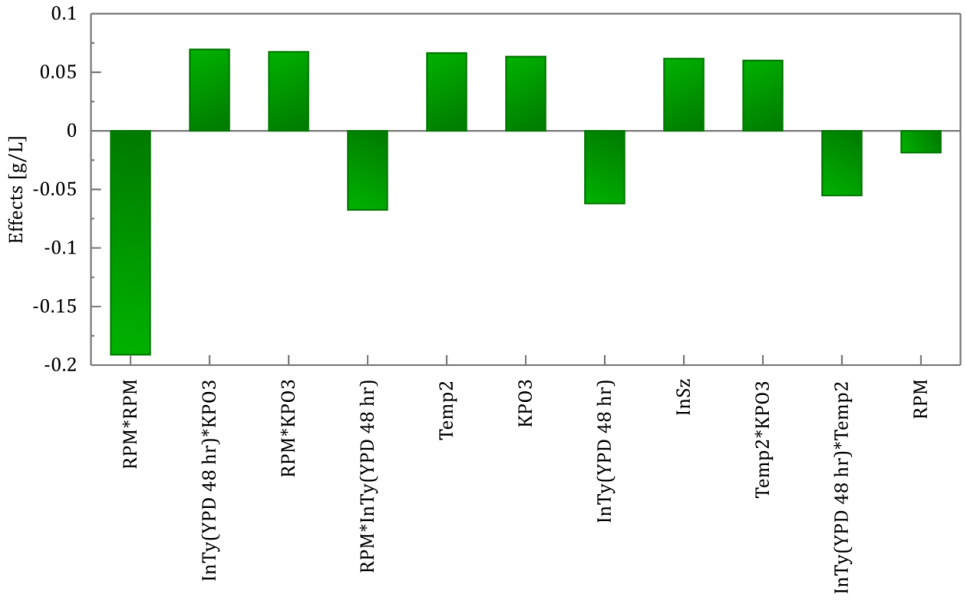


Figure 4.15. Effects of factors (and interactions (*) between factors) on the lipid yield of *M. pulcherrima* cultivated for seven days on wheat straw pretreated at 170 °C. InSz = inoculum size, InTy = inoculum type, KPO3 = potassium phosphate, YE = yeast extract.

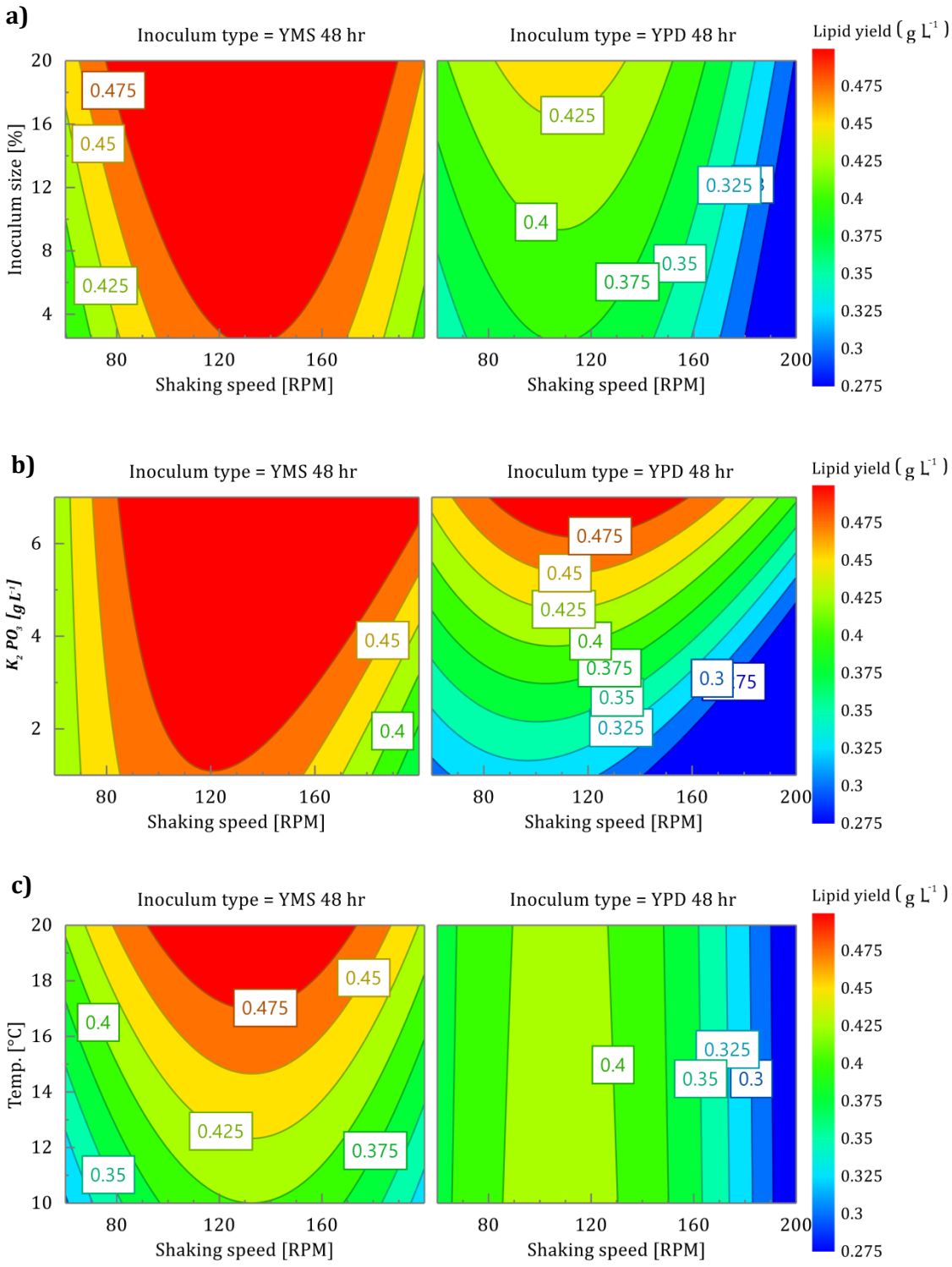


Figure 4.16. Predicted lipid yield of *M. pulcherrima* cultivated for seven days on LHW hydrolysed wheat straw. No Tween 80 and any amount of yeast extract. Inoculated with YMS (left) or YPD (right). Varying amounts of a) inoculum; b) K_2HPO_4, and c) temperatures with shaking speeds. Where variables are not part of the prediction, inoculum volume = 11.75% vol, temperature = 20 °C and K_2HPO_4 = 4 g L^{-1}.

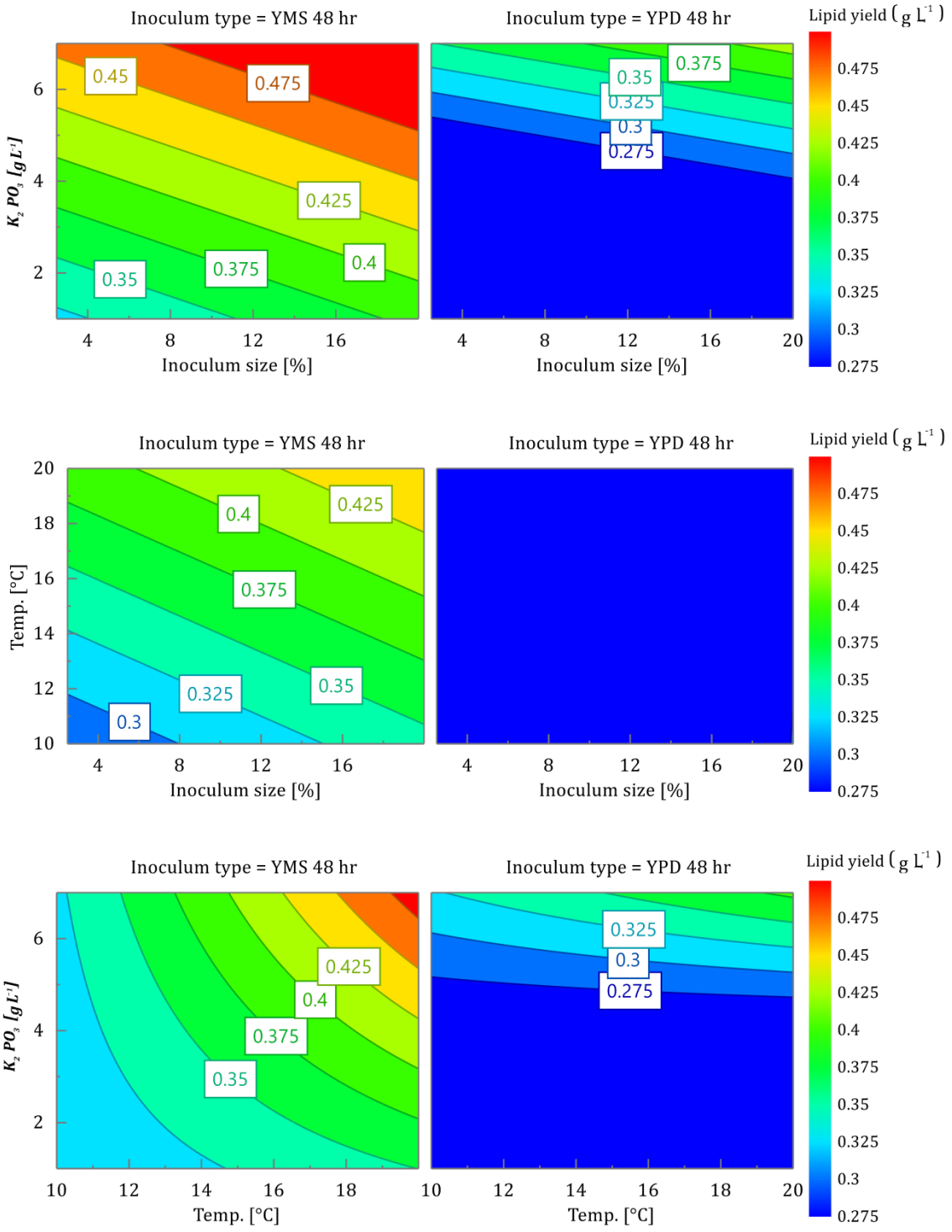


Figure 4.17. Predicted lipid yield of *M. pulcherrima* cultivated for seven days on wheat straw hydrolysed at 170 °C by a LHW flow process with no Tween 80 and any amount of yeast extract. Inoculated with either YMS (left) or YPD(right) and with varying amounts of a) K_2HPO_4, and b) temperatures with varying inoculum size and c) K_2HPO_4. Where variables are not part of the prediction, shaking speed = 200 rpm, inoculum volume = 11.75%, temperature = 20 °C and K_2HPO_4 = 4 g L^{-1}.

The model for lipid yield represents a compromise between the factors and interactions that increase the cell dry mass and those that increase the lipid content. Tween 80 and yeast extract are not significant. The positive effect of increased yeast extract concentration on the cell dry mass is offset by its negative effect on the lipid content, meaning that it has no net effect on the lipid yield.

Five variables are significant: *M. pulcherrima* performs far better when YMS inoculum is used, the temperature is higher, the maximum amount of potassium phosphate is used and the shaking speed is intermediate at around 130 rpm. These factors and trends are largely similar to the factors that are most important for increased cell dry mass.

4.4.4 Verification of model

The aim of this work was to establish whether the models generated could be used to select culture conditions that would improve the performance of *M. pulcherrima* on LHW pretreated wheat straw. Due to sample constraints, the fractional factorial design was applied directly to the wheat straw hydrolysed at 190 °C in LHW flow system without further optimisation by a full factorial design. Optimised parameters for each of cell dry mass, lipid content and lipid yield were chosen from the models defined above, with the constraints of a maximum of 10% vol inoculum, and the previously identified optimum time of 12 days when using glucose. Intermediate values were selected for variables, which were not involved in the model.

	K_2HPO_4 (g L^{-1})	YE (g L^{-1})	Tween (g L^{-1})	Temp (°C)	Shaking speed (rpm)	Inoculum type	Inoculum volume (% vol)
Cell dry mass	3.8	1	0	20	145	YMS	10
Lipid content	0	0	2	20	60	YPD	10
Lipid yield	7	0.5	0	20	145	YMS	10

Table 4.2. Variables selected for model verification on LHW pretreated wheat straw (190 °C) with nutrients of YMM with modifications to yeast extract and potassium phosphate concentration specified, pH 5, for 12 days at the temperatures specified.

The cell dry mass was increased in all samples, however the lipid content itself was decreased (Figure 4.18). The overall lipid yield was also decreased to a maximum of 0.69 g L^{-1}. This may be because the optimum fermentation time for lipid production on wheat straw hydrolysates is different from that for glucose. As the yeast had experienced a glucose-rich environment in the inoculum with no oligosaccharides present prior to being

cultured on the hydrolysate, the yeast would likely already have the enzymes present for catabolising glucose and so it would be expected to do so more rapidly.

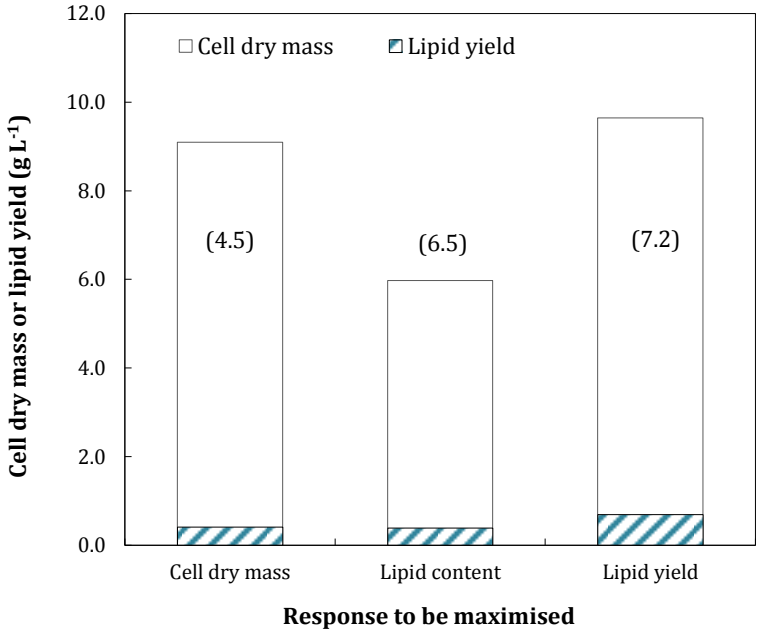


Figure 4.18. Cell dry mass (white bars), lipid yield (striped bars) and (lipid content) (%) of *M. pulcherrima* cultivated on LHW pretreated wheat straw (190 °C) under the conditions specified in Table 4.2.

In order to utilise oligosaccharides, *M. pulcherrima* may first detect their presence, synthesise the β-glucosidase, ingest the oligosaccharides or excrete the enzyme, allow it to cleave the oligosaccharides and then catabolise the resultant sugars. Additionally, the presence of glucose from either the monosaccharides produced in the hydrolysates or as the product of this enzymatic hydrolysis may repress the production of β-glucosidase. For these reasons, the rate of production of monosaccharides from oligosaccharides is likely to be slower than when only glucose is present. This means that the C:N ratio may reach a high enough level to initiate lipid accumulation at an entirely different time. Perhaps it occurs when the initial supply of glucose is depleted, as in the batch hydrolysed wheat straw. It may occur far later, when the rate of oligosaccharide depletion is at a minimum, or not at all as the concentration of glucose within the yeast cells never gets high enough.

4.4.5 Conclusions

When grown on wheat straw pretreated with liquid hot water in a batch process, *M. pulcherrima* was found to grow and produce lipid at up to 26% lipid content, with a lipid yield of 1.17 g L^{-1}. This is the first time *M. pulcherrima* has been shown to behave oleaginously on lignocellulose hydrolysate. The maximum cell mass was increased from 2

to 7 g L^{-1} by changing the hydrolysis method from batch to flow and altering the culture conditions based on a model derived from the design of experiments. The choice of inoculum type and volume was found to be crucial as it affects the resulting cell dry mass, lipid content and lipid yield as well as with what other nutrients the hydrolysate should be supplemented. The shaking speed was also found to affect the lipid content in a manner contrary to previously published literature, perhaps due to fragile cells breaking. Growth on oligosaccharides was found to be different to growth on glucose, probably because of different rates of catabolism. This affected the lipid content attainable. More research is needed into time variation and ideally monitoring the oligosaccharide and glucose content with time, and how rapidly β-glucosidase is made. A hydrolysis method that produces more glucose and does so efficiently in terms of energy input with time would be desirable.

5 Suitability of microwave hydrolysis for the cultivation of *M. pulcherrima*

5.1 Preamble: Microwave depolymerisation of cellulose

Recently, microwave irradiation has been found to hydrolyse microcrystalline cellulose effectively, without needing additional reagents or enzymes.[359, 360] Compared to hydrothermal hydrolysis by conventional heating, microwave hydrolysis is arguably more efficient achieving a greater level of cellulose solubilisation, up to fifty times higher levels of glucose, 3-4 times more cellobiose and higher ratios of glucose to other sugars and to HMF (2.8 vs. 1) over shorter timeframes. Heating systems that use microwaves are generally more efficient than conventional methods.[361, 362] Provided the reaction vessel is small enough for the microwaves to penetrate the entire sample it is heated all at once, not just the areas in contact with the heat source (though the processing does require a polar solvent). Additionally, the heat source does not need to be warmed up, enabling instantaneous heating.[361]

In cellulose, microwave irradiation (180-220 °C) was found to disrupt the hydrogen bonding between amorphous regions of cellulose and so enable auto-hydrolysis of cellulose in a SN2 reaction via levoglucosan (Figure 5.1). Temperatures above 220 °C

allow access to the crystalline region, as the hydrogen bonds between the cellulose fibres are broken. Consequently, the greatest yields of glucose (21% of theoretical) have been attained at a temperature of 245 °C (c.f. 16% by conventional heating using otherwise identical conditions.)[360] This method has the potential to be modified to a more efficient continuous flow process suitable for scale up.[362]

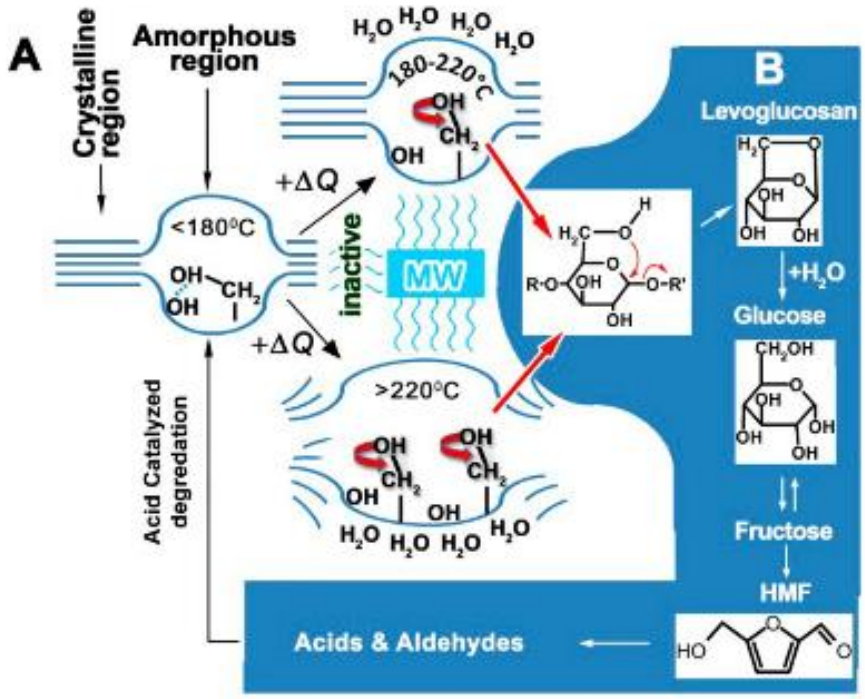


Figure 5.1. The interaction between cellulose and microwaves at temperatures between 180-220 °C and >220 °C. A) Mechanism of activation; B) cellulose degradation to acids and aldehydes via levoglucosan. Reprinted with permission from Fan *et al.*[360]

Like alternative hydrolysis methods, inhibitors are still formed from the degradation of the sugars, though in the case of microwave heating, can benefit the reaction by catalysing the hydrolysis. However, the elevated levels of inhibitors mean that species with low inhibitor tolerance such as *S. cerevisiae* cannot thrive on this hydrolysate.[363] While the majority of the research to date has used models for lignocellulose, such as cellulose, the process has been successfully attempted on Miscanthus with the addition of acid and basic catalysts, hydrolysing a maximum of ~25% of the material at 180 °C.[359] In this chapter, wheat straw will be hydrolysed using microwave hydrothermal methods at varying temperatures, ratios and holding times at these temperatures. The content of the resultant hydrolysates will be analysed, then screened for *M. pulcherrima's* ability to grow and produce lipid and other products on them. The best hydrolysis method will then be taken forward for further development.

5.2 Microwave hydrolysis products

In order to determine the microwave hydrolysis conditions that would yield the best cell dry mass and lipid content, the variables; straw to water ratio, hydrolysis temperature and holding time, were selected for screening. It was reasoned that the desired hydrolysate would have a high mono- and oligosaccharide content as well as low concentrations of inhibitors. Straw to water ratio is an important factor in providing high sugar content. Too low and it would be impossible to attain high sugar concentrations, but too high a ratio and the hydrolysate will become saturated with oligosaccharides and unable to dissolve any more.[108] Furthermore, effective convection is crucial in a microwave system to prevent the formation of hotspots that could lead to elevated inhibitor formation, whilst the rest of the straw is only partially hydrolysed, resulting in a low sugar, high inhibitor mixture that is unsuitable for yeast cultures. High straw loadings could potentially disrupt convection currents by the fibres causing blockages. The temperature is known to affect the extent of the hydrolysis and the composition of the hydrolysates and was varied to examine the yield of sugar and aid higher solubilisation.[360] The microwave took approximately five minutes to reach the set temperature. The length of time the sample is kept at that temperature before cooling (the holding time) at these temperatures was also examined (Table 5.1, and Appendix 1). This is the first report of using this method on a lignocellulosic resource as opposed to microcrystalline cellulose or waste office paper.[359, 360]

Label	Colour	Straw: water ratio	Temperatures (°C)	Holding time (min)
1:10(1)		1:10	190-200	1
1:10(0)		1:10	210-250	0
1:20(0)		1:20	180-250	0
1:20(5)		1:20	190-250	5
1:30(0)		1:30	180-210, 250	0

Table 5.1. Conditions for microwave hydrolysis of straw and the labels and colours assigned to the resulting hydrolysates. Labels assigned in the format "Ratio straw : water (holding time) Temperature °C" e.g. 1:20(0) 190 °C = straw : water 1:20, 0 min holding time at 190 °C.

The conversion of solid straw to liquid hydrolysate was calculated gravimetrically (Figure 5.2). Higher conversions increase the productivity of the entire process as less lignocellulose is wasted and the maximum yield of sugar and thus, theoretically, the yield of lipid will be higher. As anticipated, temperature was generally correlated with

increasing amounts of straw solubilised, the maximum being 59% (1:20(0) 240 °C). Higher temperatures were more effective, as above 220 °C the crystalline portions of cellulose are disrupted allowing the water to access the fibres.[360] The correlation between temperature and solubilisation was not always linear, such as in the dataset 1:20(0) and the surprisingly low yield of 1:30(0) 250 °C, which was prepared as part of a separate run to the rest of this set of hydrolysates.

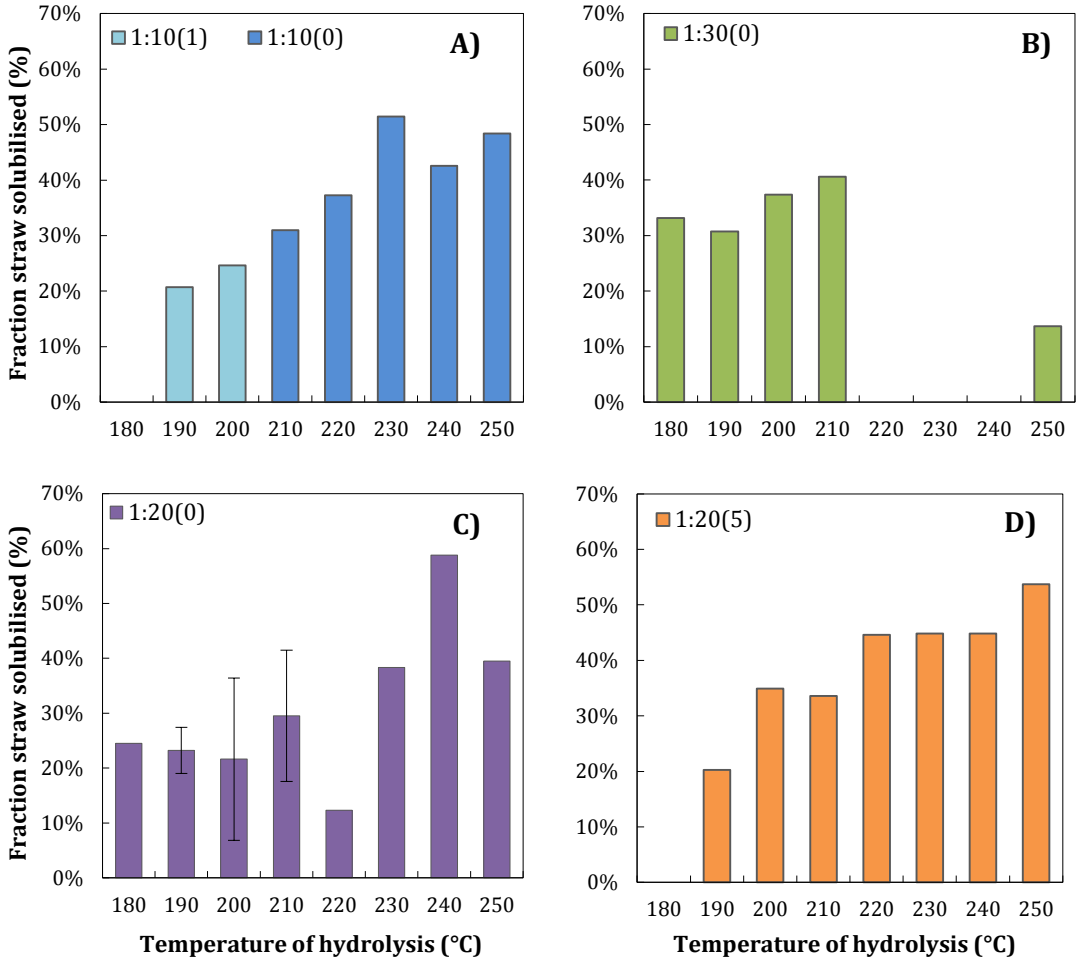


Figure 5.2. Fraction of straw solubilised after liquid hot water microwave hydrolysis at varying temperatures and holding times at a straw to water ratio of A) (light blue) 1:10(1) 190-200 °C and (dark blue) 1:10(0) 210-250 °C; B) 1:30(0); C) 1:20(0); D)1:20(5).

More intense conditions (i.e. higher temperatures and longer hydrolysis times) led to higher sugar concentrations until a maximum, after which the sugar concentration decreased. Total acid and total furfural concentrations increased approximately linearly with temperature, and do not decrease at higher temperatures.

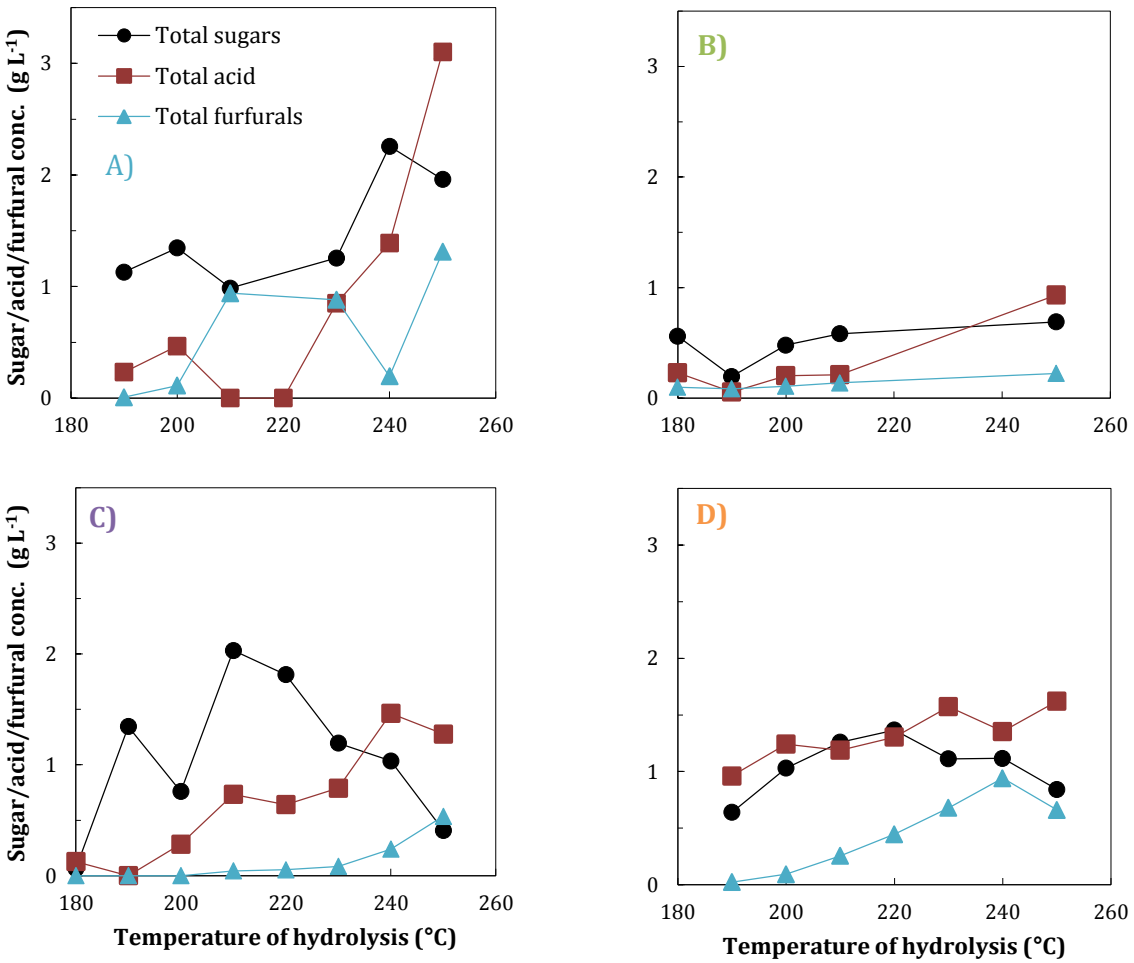


Figure 5.3. Concentration of all measured sugars (glucose, xylose, cellobiose, arabinose, rhamnose, mannose, fructose, levoglucosan, sucrose and galactose), acids (formic and acetic acid), and furfurals (furfural and 5-HMF) at a straw to water ratio and holding time of microwave hydrolysed wheat straw under the following conditions: A) 1:10(1) 190-200 °C and 1:10(0) 210-250 °C B)1:30(0) C)1:20(0) D) 1:20(5).

Consequently, as temperature is increased, there is a point at which the total concentration of inhibitors becomes greater than the total concentration of sugars (this is the case for all temperatures of the 1:20(5) series (Figure 5.3d). These observations are in accordance with the model of lignocellulose hydrolysis in which hemicellulose and cellulose are initially hydrolysed to sugars and then sugars are converted to furfurals then acids.[231] Elevated levels of all the hydrolysis products are produced at higher temperatures because rates of both hydrolysis of lignocellulose to sugar and conversion of sugar to inhibitors are faster, in addition to the involvement of crystalline portions of cellulose above 220 °C.

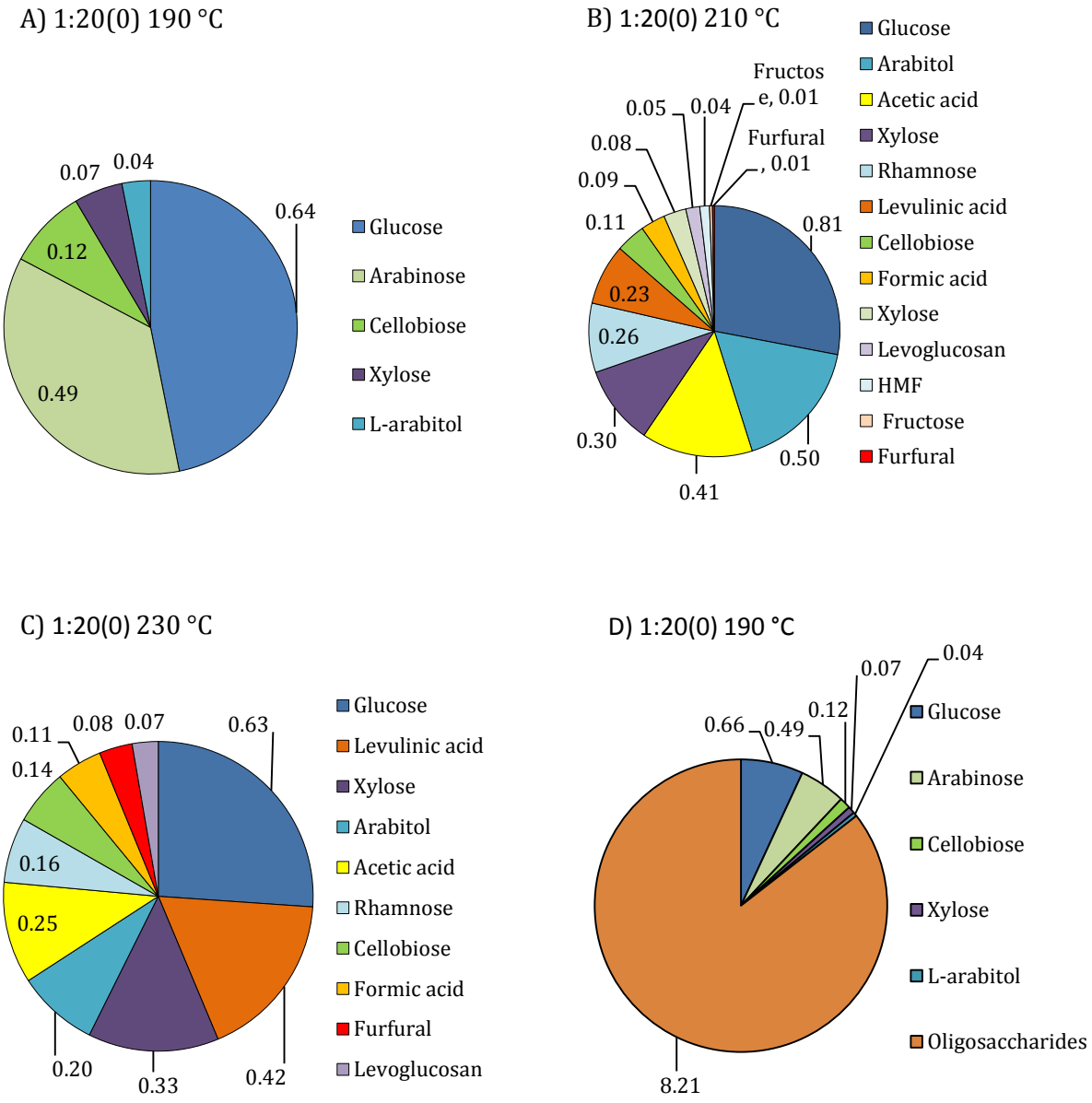


Figure 5.4. Composition of hydrolysates excluding oligosaccharides from the 1:20(0) series hydrolysed by microwave liquid hot water method at A) 190 °C, B) 210 °C, C) 230 °C; D)190 °C including oligosaccharides. All values in g L^{-1}.

The increase in furfural concentration is non-linear, which may be because having a higher concentration of sugar also further increases the rate of conversion to inhibitors (assuming sugar and acid concentrations are part of the rate equations.) Acid catalysed hydrolysis of cellulose follows first pseudo-first order kinetics[231, 232] so this seems plausible, however experiments on the initial rate of reaction would be required in order to determine the kinetics of microwave hydrolysis.

For the 1:20(0) temperature series (Figure 5.4) at a hydrolysis temperature of 190 °C, a total of 1.33 g L^{-1} monosaccharides (mostly glucose and arabinose) and no inhibitors were detectable. At a higher temperature of 210 °C, the glucose content is increased as is the range of other sugars, yet the third largest component is acetic acid, one of the breakdown products of glucose. It seems plausible that the formation of organic acids may further catalyse the hydrolysis aiding in an increased sugar yield.[359, 360]

Concentration of sugar, furfurals and acids is generally highest with a straw: water ratio of 1:10 and lowest at 1:30 (Figure 5.3A and B, respectively). Although this is expected given the concentration of straw was three times greater pre-hydrolysis, the concentration of hydrolysis products is not, indicating that the rate of hydrolysis may not be high enough to compensate for the larger amount of straw. This is confirmed by the smaller percentage of straw solubilised, for example 30% for 1:10(0) 210 °C vs. 40% for 1:30(0) 210 °C (Figure 5.2.)

The presence of xylose and rhamnose in the hydrolysate indicates that hemicellulose was accessed and broken down effectively. As well as hemicellulose, cellulose was hydrolysed as more glucose was present in the hydrolysates than xylose (Figure 4.4), whereas the hemicellulose fraction of wheat straw is comprised of approximately 75% xylose and 2% glucose.[364] This demonstrates that microwaves were still able to hydrolyse cellulose to sugar without any additional catalyst even when it was still associated with hemicellulose.

High quantities of acetic acid were also present; suggesting that hemicellulose was being further degraded in the reaction. HMF, one of the key breakdown products of glucose, is also present in some samples in higher quantities than furfural, the breakdown product of xylose. Previous studies on microcrystalline cellulose have found that microwaves yield a higher glucose to HMF ratio than conventional heating (2.8 vs. 1) and in this case, there was no HMF produced for most samples, and for 1:20(0) 210 °C and 230 °C the ratios are 3.55:1 and 1.95:1 respectively (Figure 4.4).

Compared to the hydrolysis of microcrystalline cellulose using the same microwave system,[360] wheat straw yields the maximum amounts of glucose at lower temperatures (190-210 vs. 250 °C). This is probably because of the presence of hemicellulose which is easier to depolymerise, and these results are similar to those obtained for the acid/alkali catalysed microwave hydrolysis of Miscanthus,[359] in that low yields of a range of pentoses were obtained, with some glucose also being formed under acidic conditions. Microcrystalline cellulose hydrolysates also have a lower glucose content and higher HMF

content at longer holding times. The published studies also show high selectivity for glucose over other sugars, and in this study, glucose was also the major sugar in the majority of hydrolysates. The 1:20(5) series was an exception to this presumably because, once generated, more of the glucose was converted to inhibitors over the longer five minute holding time.

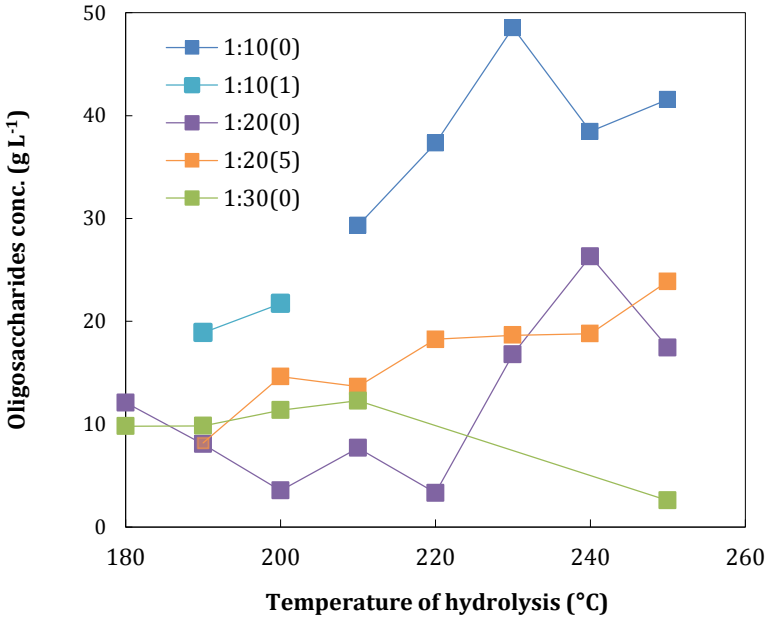


Figure 5.5. Concentration of oligosaccharides in microwave hydrolysed wheat straw hydrolysates by mass balance.

Given that the total amounts of quantifiable mono- and disaccharides and inhibitors were in all cases lower than the total mass of solubilised straw, the remaining mass solubilised was assumed to be oligosaccharides, though this could also include solublised lignin.[232] An example of the amount of oligosaccharides compared to the amount of mono and di-saccharides is shown in Figure 4.4d. The concentration of oligosaccharides varies between 2.5 and 48 g L^{-1}, with more intense conditions having a higher concentration of oligosaccharides (Figure 5.5).

5.3 *M. pulcherrima* cultures on microwave hydrolysates

The growth and lipid production of *M. pulcherrima* on the microwave hydrolysates were determined gravimetrically after seven days cultivation at 25 °C. (Figure 5.6 and

Figure 5.8). The growth of *M. pulcherrima* was better on straw hydrolysed under less intense conditions despite there being lower sugar and oligosaccharide concentrations in these samples. An example of this is the difference between the holding times for 1:20(0) vs 1:20(5). (Figure 5.6 C and D respectively) with the longer holding times having poorer growth. Clearly holding time is an important factor. Higher hydrolysis temperatures led to less growth, most notably in Figure 5.6 B, C and D at temperatures over 210 °C, which is when cellulose starts to be degraded.

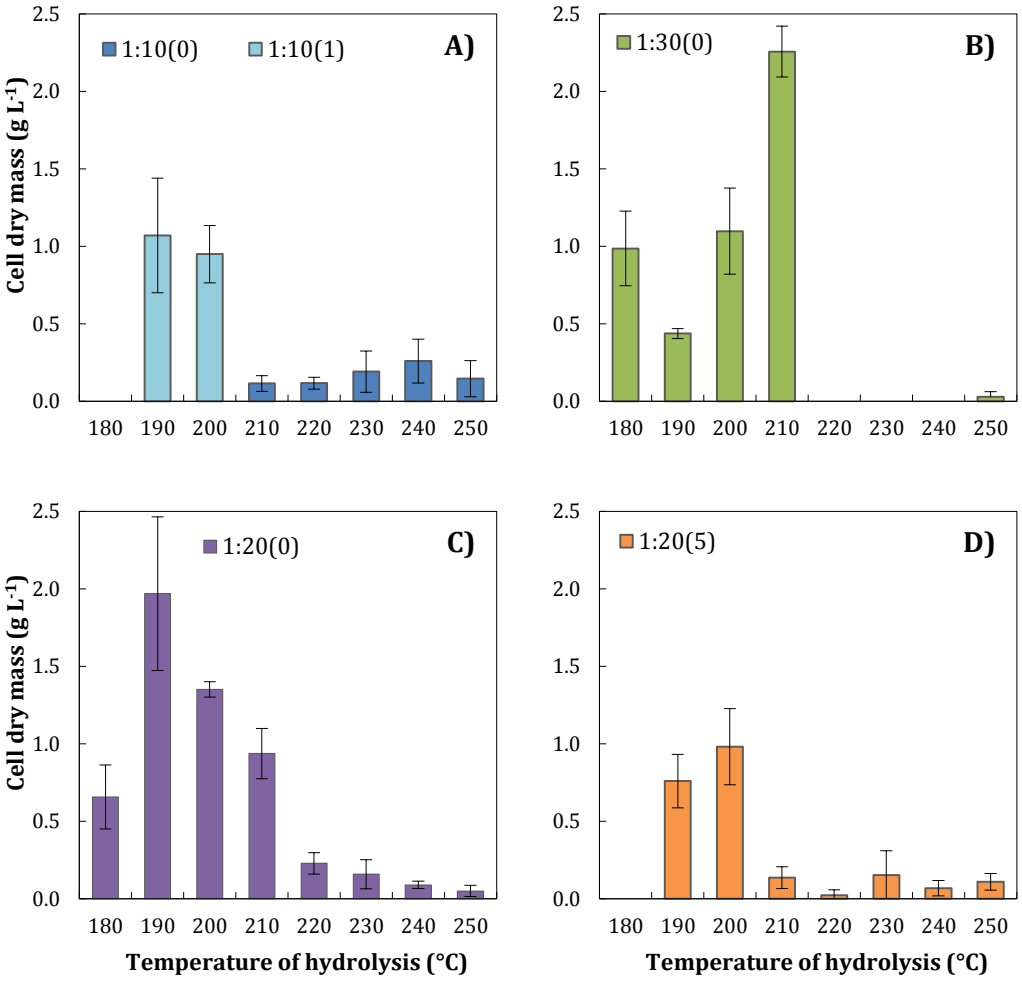


Figure 5.6. Cell dry mass of *M. pulcherrima* grown at 25 °C, 180 rpm, for seven days on microwave hydrolysed wheat straw, hydrolysed at a straw: water ratio of A) 1:10(1) (light blue) and 1:10(0) (dark blue) B) 1:30 with no holding time C)1:20(0), D) 1:20(5).

Finally, higher loadings of straw have worse growth such as 1:10 compared to 1:30. The lower growth under these more intense conditions is likely to be caused by the higher concentration of inhibitors in these samples. There is an inverse correlation between

inhibitor concentration and growth, (Figure 5.7A) in which samples with a higher overall concentration of inhibitors than sugars yield a negligible cell dry mass. Although *M. pulcherrima* is reasonably tolerant of inhibitors, the combination of all the inhibitors in the hydrolysate probably led to a synergistic inhibitory effect. Nevertheless, that *M. pulcherrima* was able to grow at all when the concentration of inhibitors was higher than or equal to that of monosaccharides is indicative of its robustness (Figure 5.7A). The best growth on microwave-hydrolysed straw (1:30(0) 210 °C, 2.26 g L^{-1}) was 60% of that obtained when *M. pulcherrima* was grown on YMM under identical conditions (3.8 g L^{-1}).

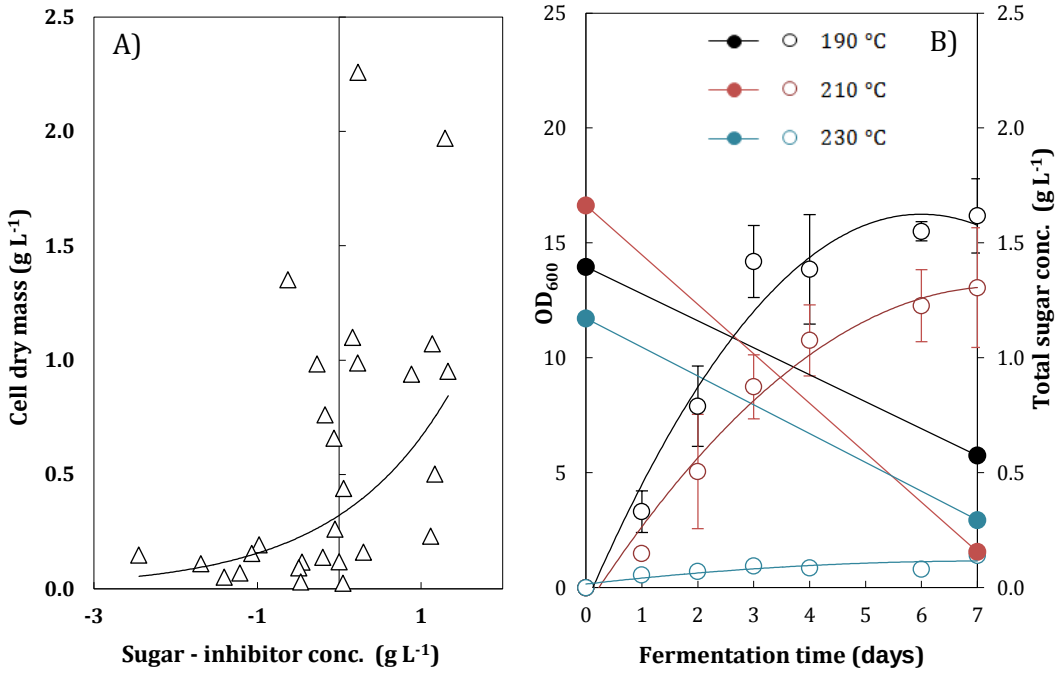


Figure 5.7 The effect of inhibitors and sugar concentration on the grown of *M. pulcherrima* in microwave liquid hot water wheat straw hydrolysates A) Cell dry mass of *M. pulcherrima* grown (7 days 25 °C, 280 rpm) vs. the difference between sugar concentration and inhibitor concentration. B) Fermentation profile for 1:20(0) 190 °C (black), 210 °C (red), and 230 °C (blue) including optical density (OD_{600nm}, empty circles) and sugar concentration(filled circles.)

In 1:20(0) 190 °C, which was one of the hydrolysate which demonstrated the best growth, there was a lag time of three days to reach stationary phase. The control with 30 g L^{-1} glucose and no inhibitors (Figure 5.7b) had the same lag time. At higher temperatures of hydrolysis (210 and 230 °C), the lag time increased and the overall cell dry mass decreased. These hydrolysates contained more inhibitors and monosaccharides. The amount of sugar utilised and the rate of sugar catabolised varies little between these three

hydrolysates, indicating that sugar uptake is probably not the rate-limiting step. In the more intensely hydrolysed, high inhibitor solution (1:20 (0) 230 ° C), it may be that the yeasts are using the sugar merely to survive rather than reproduce.

The samples which yielded the most biomass also generated the greatest lipid content, with 1:10(1) 190 °C, 1:20(0) 190 °C and 1:30(0) 210 °C having lipid contents in (or very close to) the oleaginous range (Figure 5.8).

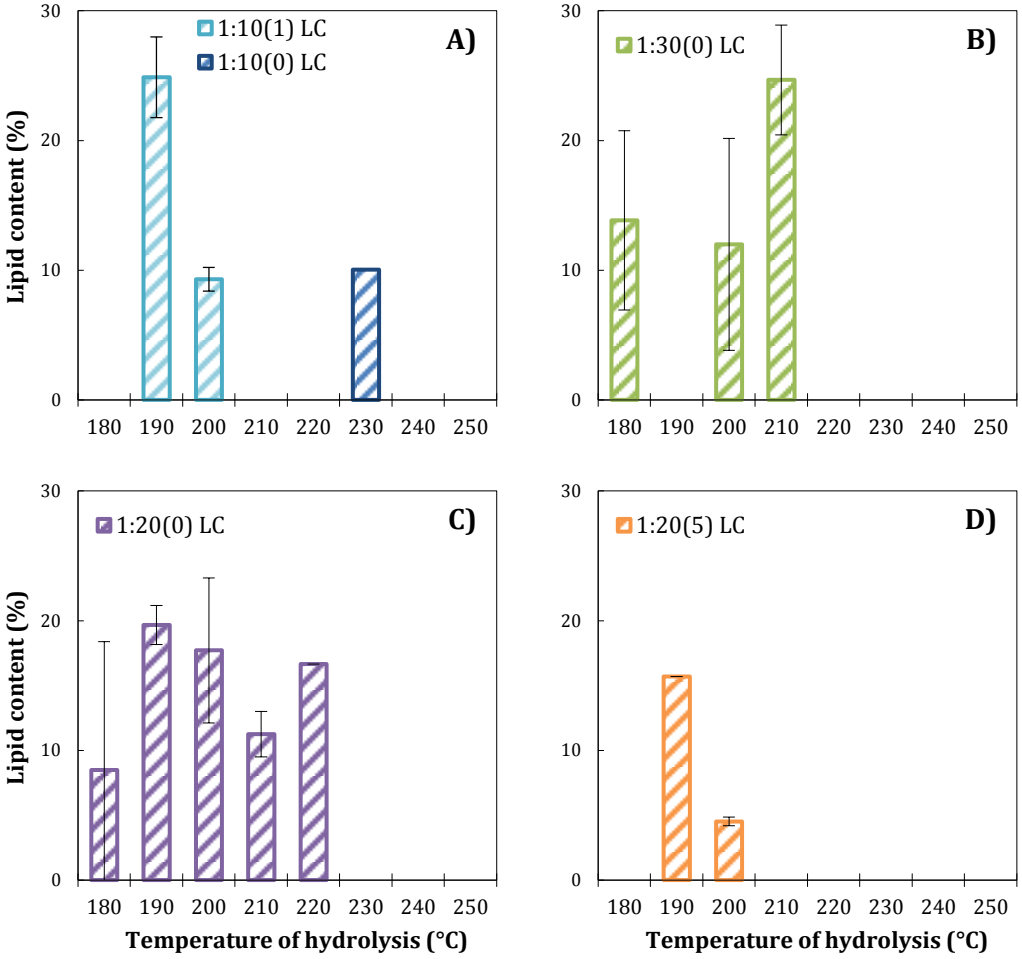


Figure 5.8. Lipid content of *M. pulcherrima* grown at 25 °C for seven days on microwave hydrolysed wheat straw, hydrolysed at a straw: water ratio of A) 1:10(1) (light blue) and 1:10(0) (dark blue) B) 1:30 with no holding time C)1:20(0), D) 1:20(5). The lipid content was determined for all samples that yielded enough biomass (over 0.02 g /0.5 g L^{-1}) to obtain this measurement. Not all replicates yielded sufficient biomass, and so not all lipid content data has error bars associated with it.

This is probably because inhibitor concentrations in these samples are lower and the yeast is able to access enough sugar that the C:N ratio was sufficiently high to trigger lipid

accumulation. Although the sugar content was lower than the control medium, the control medium had an average lipid content of 11% as the temperature was kept at 25 °C whereas lower temperatures initiate lipid production. This suggests that stress mechanisms other than low temperature may be preventing sporulation, possibly the higher inhibitor content or the low sugar conditions.

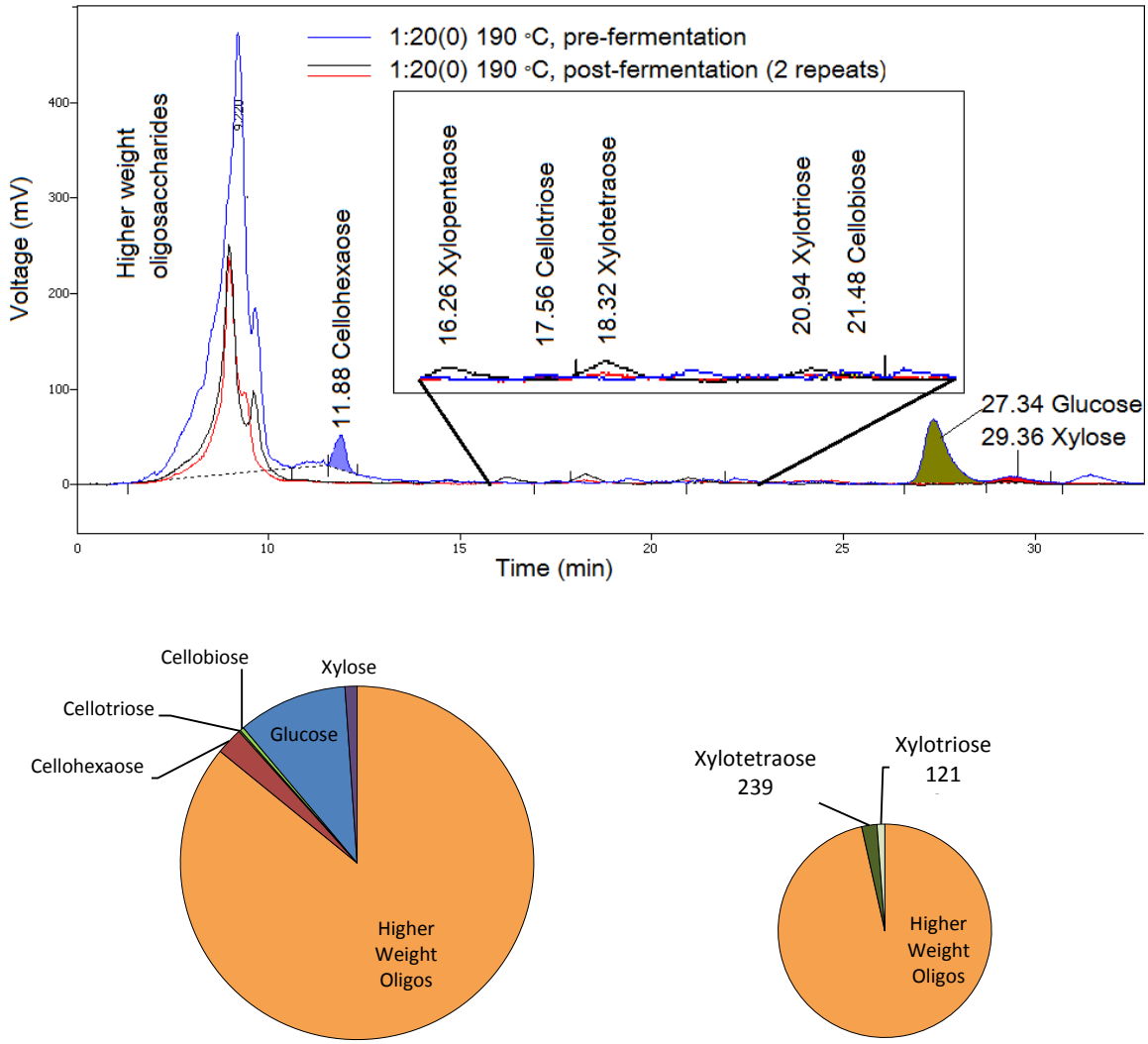


Figure 5.9. A) HPLC trace of 1:20(0) 190 °C before (blue) and after fermentation (two repeats, black and red) by *M. pulcherrima* run on a Hi-Plex Na column designed for separating oligosaccharides. B) Proportions of oligo- and monosaccharides before fermentation (left) and after fermentation (right). Areas of charts are proportional to total area under chromatogram peaks.

The cell dry mass yield from the sugar input was in many instances higher than the amount of sugar consumed.-in the case of 1:20 (0) 190 °C, there were 2.55 g of yeast produced for every gramme of sugar consumed. This conversion is over eight times

higher than the maximum theoretical yield of biomass from the monosaccharide sugars. This indicates that the yeast is able to metabolise the oligosaccharides. To assess the types of oligosaccharides being metabolised, the fermentation hydrolysate before and after fermentation (1:20(0) 190 °C) was assessed using a Hi-Plex Na HPLC column capable of separating oligo- and mono-saccharide sugars (Figure 5.9).

While the concentration of oligosaccharides in this sample is low compared to the other hydrolysates (Figure 5.5) there was a 39% reduction in the area of higher weight oligosaccharide (7-10 monosaccharide units long) peak (elution time 6-10 min) after fermentation with *M. pulcherrima* (Figure 5.9A). Additionally, all cellohexose, cellotriose and cellobiose were consumed on fermentation. No significant production of shorter chain oligosaccharides was observed. This suggests that *M. pulcherrima* is capable of moving the oligosaccharides in to the cell through endocytosis and metabolising internally and not producing external enzymes that cleave the saccharides into smaller chain oligomers. Alternatively, if the oligosaccharides are cleaved externally to the cell, then the resulting smaller chains are rapidly consumed by *M. pulcherrima*; *C. curvatus* is thought to carry out endocytosis on oligocelluloses,[251] and *M. pulcherrima* may have evolved a similar mechanism.

However, this is not the case with some of the oligoxyloses. No short chain oligoxyloses are present before fermentation yet small quantities of xylotetra

ose and xylotriose were observed afterwards. This may be due to the yeast hydrolysing the longer chain oligoxyloses within the cell and ejecting the saccharides it cannot catabolise, or that external enzymes can cleave the oligoxylose chains but the yeast in unable to metabolise the resulting small chain sugars. As creating enzymes for this function would be atypically wasteful, it seems more likely that the utilisation of these xylose-based sugars is slower, perhaps because of glucose repression or a similar mechanism.

Alternatively, extra-cellular hydrolysis of higher weight oligosaccharides produces both glucan and xylan, as although cellulose is exclusively made from glucose, hemicellulose includes glucose as well as xylose,[364] and the glucose is used preferentially over the xylose.[184]

A similar amount of sugar is initially available and consumed in the 1:20(0) 230 °C, and 1:20(0) 190 °C hydrolysates yet the growth is much lower at the higher temperatures (Figure 5.6). It therefore may be that the higher level of inhibitors in the 230 °C sample

(Figure 4c and Figure 5.3) are inhibiting the ability of *M. pulcherrima* to utilise oligosaccharides. There is no correlation between mono- or oligosaccharide concentration and growth, even at total inhibitor concentrations under 1 g L^{-1} indicating that as monosaccharide concentration is so low there is not a significant enough difference between the sugar concentrations accessible to *M. pulcherrima* in order for it to behave differently (see Appendix 2). This suggests that the hydrolysis of oligosaccharides is either too slow to be used for growth in the seven day timeframe or are not used by *M. pulcherrima* for growth

5.4 By-product formation by *M. pulcherrima*

One method of reducing the cost of a microbial palm oil replacement is to co-produce value chemicals alongside.[208, 365] For example, *M. pulcherrima* is known to produce both arabitol and 2-phenylethanol in small quantities in the wine fermentation (Figure 5.10).[366] Arabitol is produced by many yeasts in the *Candida* clade,[367] and is one of twelve C3-C6 carbon building blocks targeted for further biotechnological research by the U.S. Department of Energy.[367] In yeasts it is produced from arabinose through a one-step reduction by an aldose, though this pathway can go on to xylulose 5-phosphate. Arabitol is currently used as an artificial sweetener and adipose tissue reducer as well as a substrate in the production of several chemicals including ethylene glycol, xylonic acid, and propene,[367] all of which are suitable building blocks for the production of bioplastics.[367]

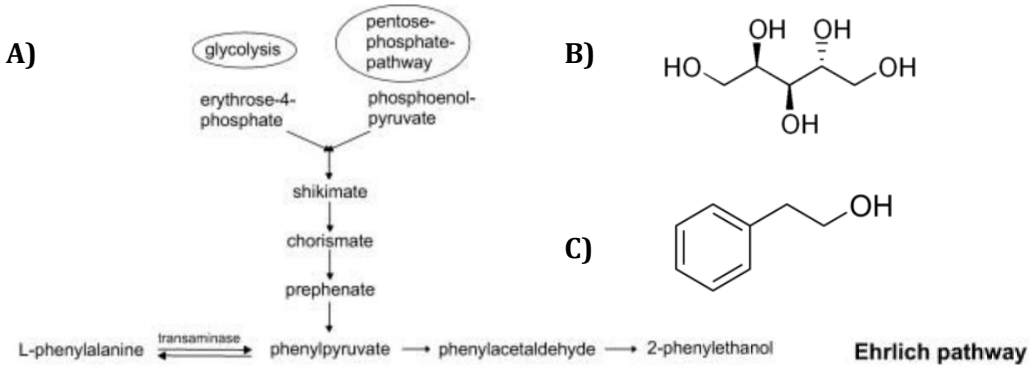


Figure 5.10 By-products of *M. pulcherrima* cultivations. A) The Ehrlich pathway found in yeast for conversion of L-phenylalanine to 2-phenylethanol; B) Arabitol; and C) 2-phenylethanol. The Ehrlich pathway begins with phenyl pyruvate, which can either be derived from 2-phenylalanine by a transaminase or by de-novo synthesis from the products of glycolysis (erythose-4-phosphate) and pentose-phosphate pathway (phosphoenol pyruvate). [368]

2-Phenylethanol (2PE) is a rose-scented aromatic compound, which is currently produced for the perfume and food industries.[369] Approximately 7000 tonnes is manufactured globally. While the majority of 2-phenylethanol is produced via the Friedel-Crafts reaction of benzene,[370] this is not suitable for food grade production, where the compound is extracted from rose petals. Due to the high costs of the biological route, a microbial source is eagerly sought.[367] Yeasts are known to produce 2-phenyl ethanol by the Ehrlich pathway (Figure 5.10 c.) The alternative pathway for 2-phenyl alanine (the cinnamate pathway, not shown) feeds into the tricarboxylic acid cycle. The first enzyme in this pathway that converts 2-phenylalanine to trans-cinnamate is repressed by glucose in *R. glutinis*.[368]

To determine the suitability of co-product formation the arabitol and 2-phenylethanol content of the post-culture broths was also determined by HPLC (Figure 5.11). The arabitol production was highest in the 1:10(1) fermentation for both 190 °C and 200 °C hydrolysis temperatures, producing up to 0.7 g L^{-1}, with all other samples producing approximately 0.2 g L^{-1}. In terms of total sugar and inhibitors, the 1:10(1) samples were most similar to 1:20(0) 190 °C, though this run only produced approximately 0.5 g L^{-1} arabitol. The differences between these two samples were that the 1:10(1) samples had higher oligosaccharide contents and more xylose than glucose. This suggests that elevated quantities of xylose relative to glucose, or higher oligosaccharides are beneficial to arabitol production. The lipid content was also relatively high for these samples (25%), suggesting it is possible to combine lipid and arabitol production.

2-phenylethanol production correlated strongly with yeast biomass, with the highest levels of 2-phenylethanol being observed with the best *M. pulcherrima* growth. This suggests that this is a product that *M. pulcherrima* makes under lower stress conditions. The content of both arabitol and 2PE was lower than when *M. pulcherrima* is cultured on glucose, suggesting that glucose is vital for the functioning of these pathways. At these titres, it is unlikely that this would be effective in improving the economics of a lipid producing process. The yield of 2-PE could be improved by the addition of L-phenylalanine to encourage the pathway utilising this amino acid, as then this is the sole nitrogen source; the Ehrlich pathway is favoured over the non-2-phenylethanol producing cinnamate pathway. Higher glucose concentrations may also suppress the cinnamate pathway. For de-novo synthesis without 2-phenylalanine, the sugars present in the wheat

straw hydrolysate (xylose, arabinose) may not produce enough of the right intermediates, so higher glucose concentrations would help here also.

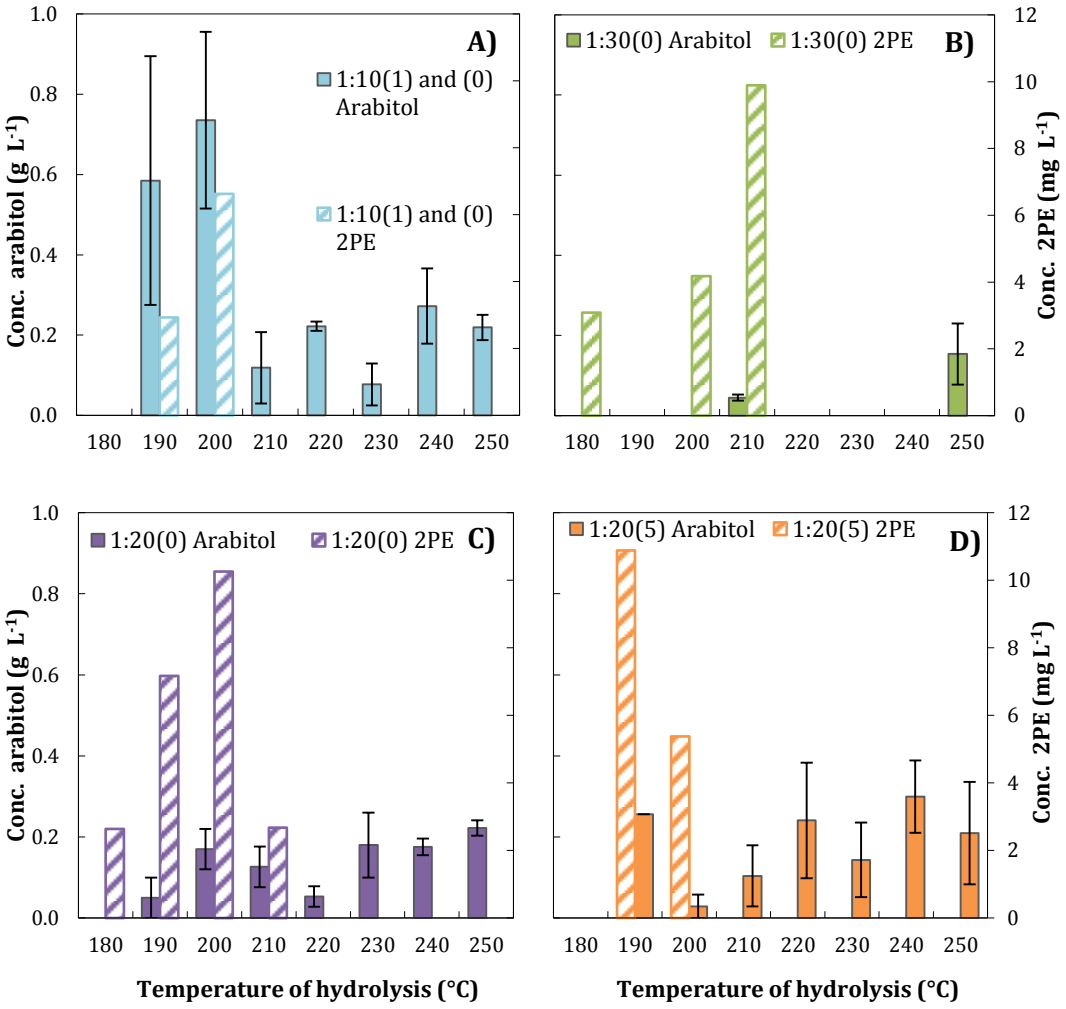


Figure 5.11. Content of arabitol (solid bars) and 2-phenyl ethanol (2PE, striped bars) after growth of *M. pulcherrima* (7 days, 25 °C, 180 rpm) microwave hydrolysates, hydrolysed under the following conditions. A) 1:10(1) and 1:10(0) B) 1:30 with no holding time C) 1:20(0), D) 1:20(5). Only samples with growth over 0.5 g L^{-1} were tested for 2PE content

5.5 Improving the lipid yield on microwave hydrolysate

The overall lipid yield was low compared to other yeast grown on lignocellulose hydrolysate that produce cellulases (Table 5.2), this is presumably due to the low biomass yield, though the cellular lipid content was also reduced when compared to simpler carbon sources such as glycerol (47 dry weight %, Chapter 2). The low biomass yield was likely to be due to the presence of inhibitors and the stress of growing in low nutrient conditions,

necessitating the generation of cellulases. 1:30 (0) 210 °C was the most successful hydrolysate and perhaps slightly outperformed the control medium in terms of lipid yield.

Hydrolysis conditions	T (°C)	Lipid yield ($g\ L^{-1}$)	Lipid coefficient ($mg\ g^{-1}$)	Lipid productivity ($g\ L^{-1}\ d^{-1}$)
M. pulcherrima, wheat straw, microwave hydrolysis				
1:10(1)	190	0.26	3	0.04
	200	0.9	1	0.01
1:10(0)	230	0.2	0.2	0.003
1:20(0)	180	0.6	1	0.01
	190	0.39	8	0.06
	200	0.24	5	0.03
	210	0.11	2	0.02
	220	0.4	1	0.01
1:20(5)	190	0.12	2	0.02
	200	0.4	1	0.01
1:30(0)	180	0.14	4	0.02
	200	0.13	4	0.02
	210	0.56	17	0.08
M. pulcherrima, control medium, chemically defined				
NA		0.47	NA	0.08
C. curvatus , corn stover, alkali pretreatment then enzymatic hydrolysis.[157]				
NA		15.1	98	4.69
Microsphaeropsis sp, wheat straw, steam explosion, no enzymatic hydrolysis.[250]				
NA		NA	42	NA

Table 5.2. Efficiency of lipid production by *M. pulcherrima* and two comparable studies. Lipid yield = mass of lipid per litre of medium, lipid coefficient = mass of lipid per mass of starting material; lipid productivity = mass of lipid produced per litre of medium per day of fermentation.

The lipid yield and productivity of *M. pulcherrima* on straw is far lower than that of two comparable studies. *Microsphaeropsis sp.* also produces cellulases and was grown in a solid-state fermentation on wheat straw hydrolysed without enzymes yet yielded more than double the best lipid yield of *M. pulcherrima* (42 vs. 17 mg g^{-1}).[250] *C. curvatus* also produces cellulases[157, 251] and outperformed *M. pulcherrima* to a great extent on every measure, although it was grown on a different substrate that was hydrolysed by additional enzymes.

In order to improve the lipid yield of *M. pulcherrima* several routes were considered. The carbon to nitrogen ratio must be balanced so as to allow *M. pulcherrima* to grow and reproduce, yet in order to accumulate lipid, must be low enough for nitrogen to be entirely consumed by the time the stationary phase is reached. Given that the limiting factor appeared to be the cell dry mass more than the lipid content, by comparison to the control, it was concluded that additional nutrients might help to boost the growth. This would presumably enable *M. pulcherrima* to produce enzymes more easily, including any cellulases or xylanases, and to lower the stress in the lag and exponential phase, perhaps also increasing the productivity.

Zhao *et al.* found that the addition of 0.5 g L^{-1} ammonium sulfate doubled the lipid yield of *C. curvatus* when cultivated on corn stover hydrolysate, and it was able to metabolise more of the sugars as a consequence.[157] While ammonium sulfate would increase the cost of process similar alternative waste resources exist. For example, rapeseed meal (RSM) is a waste product in the existing edible oils industry that is rich in protein, as well as carbohydrates and other nutrients. Subsequent to biological pretreatment with fungal spores and autolysis at 72 h^{-1} and 55 °C, it has been used to supplement the growth of *Rhodosporidium toruloides* on glucose. Under fed-batch conditions, this yeast yielded a cell dry mass, lipid content, and lipid yield of 77.7 g L^{-1}, 54.4% and 42.3 g L^{-1} respectively – this is a comparable lipid production to the culture that used yeast extract instead of RSM. This nutrient source was therefore selected for further investigation and was hydrolysed by microwave heating.

The previous experiments were conducted for seven days at 25 °C, under which conditions the control medium also produced a low lipid content of 11%, as expected as the process conditions were therefore modified in accordance with the model derived in Chapter 3 under high oxygen and low oxygen conditions.

Label	Carbon source	Time (days)	T[a] (°C)	T[b] (°C)	Inoculum Type	Inoculum volume (%)	Nutrient source	Shaking speed (rpm)
C0	Glucose	7	25	25	YPD	2.5	YMM	180
WS0	Wheat straw	7	25	25	YPD	2.5	YMM	180
C1	Glucose	12	25	20	YMS	5	YMM	150
WS1	Wheat straw	12	25	20	YMS	5	YMM	150
C2	Glucose	12	25	20	YMS	5	RSM	150
WS2	Wheat straw	12	25	20	YMS	5	RSM	150
C3	Glucose	12	25	10	YMS	5	YMM	0
WS3	Wheat straw[c]	12	25	10	YMS	5	YMM[d]	0

Table 5.3. Conditions for improving the lipid yield of *M. pulcherrima* on microwave hydrolysed wheat straw (1:20(0) 190 °C) compared to glucose (30 g L^{-1}). Nutrient source was either the nutrient composition of yeast minimal medium (YMM) or rapeseed meal (RSM) hydrolysed by microwave at 190 °C at a water : RSM ratio of 1:20, combined with carbon source at a 1:4 ratio. a) Temperature for first three days; b) temperature for subsequent days; c) microwave hydrolysed wheat straw 1:20(0) 190 °C 200 °C 210 °C ; d) YMM nutrients added, with K_2HPO_4 concentration reduced from 7 g L^{-1} to 3.46 g L^{-1}

Interestingly, the conditions which had improved the cell dry mass and lipid content of the conventionally hydrolysed wheat straw (

Figure **5.12**, WS1, 1.82 g L^{-1}, 11 ±4%) made no significant change to the cell dry mass and decreased the lipid content compared to the original conditions (WS0, 1.96 L^{-1}, 19 ±2%). This appears to be an issue with the wheat straw component of the medium as the control medium increased in both cell dry mass and lipid content. Adding rapeseed meal in place of nutrients improved the cell dry mass of both the control and the wheat straw sample (C2, 7.2 vs 4.7 g L^{-1}; WS2, 2.7 vs 1.8 g L^{-1}) but did not improve the lipid content of the control and even lowered it in the wheat straw case, meaning that the lipid yield was not improved. This suggests that RSM hydrolysed by microwave provides some nutrients including nitrogen, but that the nutritional balance of the wheat straw hydrolysate is unsuitable for lipid production at this loading. The inhibitors and low monosaccharide content in the wheat straw hydrolysate are also likely to be limiting its growth.

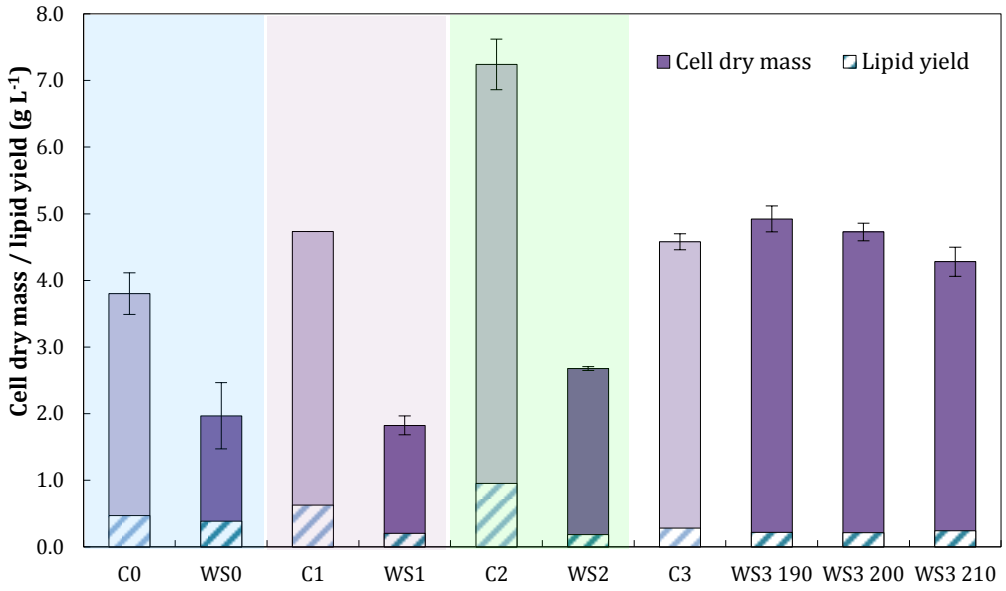


Figure 5.12. Varying the culture conditions of *M. pulcherrima* grown on liquid hot water microwave hydrolysed wheat straw in an attempt to improve the lipid yield. Conditions are as per (Table 5.3). Cell dry mass (solid bars) and lipid yield (striped bars) of wheat straw hydrolysates (darker), compared to control media (lighter) with glucose as the carbon source. Culture conditions for each label are indicated by the coloured backgrounds.

Element	Concentration (mg L^{-1})	Element	Concentration (mg L^{-1})
Sodium	20.8	**Calcium**	150.0
Magnesium	44.6	**Manganese**	0.9
Silicon	249.1	**Iron**	0.6
Phosphorus	19.6	**Copper**	0.1
Sulfur	0.0	**Zinc**	0.3
Potassium	693.6	**Barium**	1.1

Table 5.4. Concentration of trace elements in microwave hydrolysed what straw sample 1:20(0) 190 °C. Determined by inductively coupled plasma mass spectrometry.

One explanation for the reduced lipid content was that the C:N ratio was too high under the new conditions and so nitrogen starvation did not occur. We therefore examined the elemental composition of the wheat straw hydrolysate (WS0) by ICP and CHN analysis (Table 5.4). The C:N ratio was found to be 32, which is lower than that found in the

standard YMM (58). As not all of the oligosaccharides are available for catabolism and some of the carbon may be in the form of aromatic compounds from lignin, the C:N ratio is likely to be even lower.

That the additional nutrients caused the decreased lipid content was verified by adding just YMM nutrients and not varying any other parameter from WS0, which yielded a decreased lipid content of 15% vs. 19 % in the original sample and a decreased cell dry mass of 1.3 g L^{-1}. That this lipid content is lower than that of WS1 suggests that the model derived from the conventionally hydrolysed wheat straw is not applicable to the microwave hydrolysed wheat straw.

5.5.1 Cultivation on microwave hydrolysed seaweed

Another feedstock of interest to the biotechnology industry is seaweed, as this also does not compete for agricultural or forest land, is fast growing without requiring freshwater or temperature control, converts more light energy to chemical energy than terrestrial crops and collection costs are low[230,371] In addition to the naturally growing seaweed, approximately 27 million metric tonnes of seaweed are cultivated every year.[371]

Seaweed (*Fucus vesiculosus,* commonly known as bladder wrack) has been shown to be hydrolysed into sulfated sugars by microwave hydrolysis.[64] This method was adapted, and the hydrolysate used to cultivate *M. pulcherrima* for between 7 and 14 days at 25 °C, as cultivation time had been hypothesised as a limiting factor for lipid production (Figure 5.13). The lipid content was higher after 14 days, and so to further improve the lipid yield, nutrients were added including increasing amounts of ammonium sulfate, and supplemental calcium chloride, magnesium and phosphate. To increase the nitrogen content rapeseed meal and jellyfish, an abundant (ca. 0.26 ±2.3 kg ha^{-1} around Ireland) marine based nitrogen source[372], were added as non-chemically defined nutritional supplements, and were microwaved simultaneously with the seaweed. The cell dry mass varied little for each of the additional nutrients compared with the samples without nutrients, although RSM (10 g L^{-1}) and yeast extract (10 g L^{-1}) were perhaps the best at 4.22 and 4.26 g L^{-1} respectively. This suggests that nitrogen is not the limiting factor for *M. pulcherrima*'s growth on seaweed. The lipid content was more variable, with 1 g L^{-1} ammonium sulfate producing a higher lipid content than 0 g L^{-1}. 2.5 g L^{-1} ammonium sulfate had the lowest lipid content of this series. This is in line with the hypothesis that some nitrogen helps the yeast grow and produce enzymes, but too much and lipid accumulation is not initiated. The best lipid contents were achieved through the addition

of rapeseed meal (26.7%), followed by jellyfish (23.4%). These probably contain a near optimal level of accessible nitrogen to ensure growth and lipid accumulation, and perhaps supplemental nutrients to help the yeast accumulate lipid.

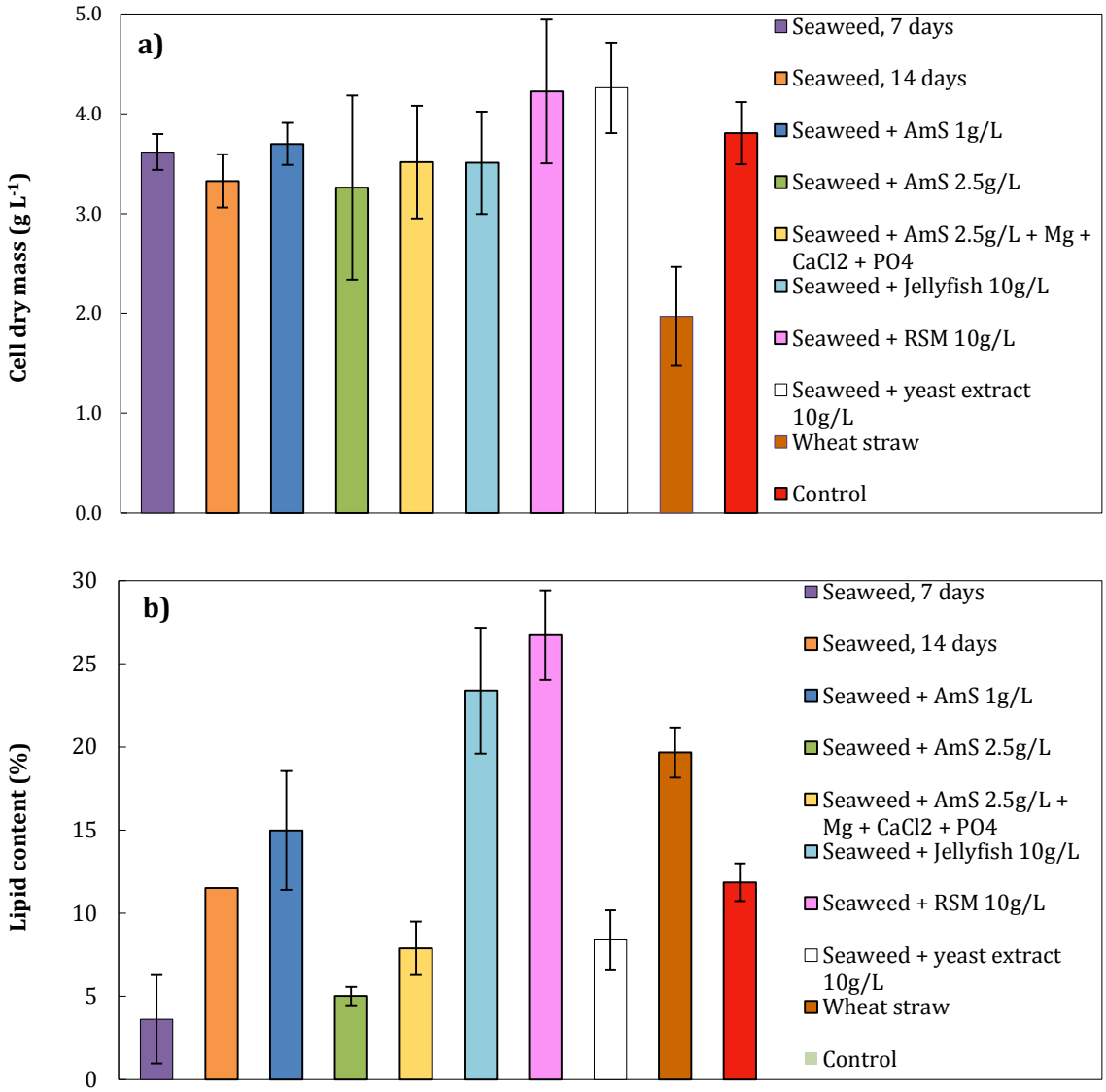


Figure 5.13. a) Cell dry mass and b) lipid content of *M. pulcherrima* grown for 14 days (unless otherwise specified) on microwave-hydrolysed seaweed with additional nutrients, and on wheat straw and YMM control medium for seven days as a comparison. AmS = ammonium sulfate, RSM = rapeseed meal, Mg = MgSO4 1g L^{-1}), PO4 = Na_2HPO_4 1g L^{-1}, CaCl2 = $CaCl_2$ 0.15g L^{-1}.

The maximum cell dry mass (4.26 vs. 1.97 g L^{-1}) were greater than wheat straw hydrolysed by the same method cultured under the same conditions and therefore signifies that seaweed is a more promising feedstock for *M. pulcherrima* cultivation than

straw. The optimal lipid content and cell dry mass were obtained when rapeseed meal was added, leading to a lipid yield of 1.12 g L^{-1}. This is similar to the lipid yield of *R. glutinis* on concentrated acid hydrolysed wheat straw (1.4 g L^{-1}),[163] yet not as good as *R. gramnis* on corn stover (16 g L^{-1}).[127] This is, however, the first time that an oleaginous yeast has been cultivated on a seaweed hydrolysate. Such hydrolysates are usually rich in sulfated polysaccharides, further demonstrating the breadth of sugar catabolism in this species.

5.6 Conclusions

This chapter demonstrated the use of un-catalysed hydrothermal microwave hydrolysis on lignocellulose led to the partial hydrolysis of both hemicellulose and cellulose, yielding a maximum of 58% solubilisation of the straw, and a maximum of 2.26 g L^{-1} monosaccharides. A range of sugars were produced, with glucose being the predominant monosaccharide. Sugar concentration and straw conversion were correlated with higher temperatures and longer holding times, up to a maximum point, after which the sugar concentration decreased as it was converted to inhibitors.

There was further evidence of *M. pulcherrima* utilising oligosaccharides, possibly within the cell, as opposed to excreting cellulases into the media broth. The most effective hydrolysis conditions in terms of cell dry mass and lipid content of *M. pulcherrima* were found to be 1:20(0) 190 °C and 200 °C, 1:10(1) 190 °C and 1:30(0) 210 °C. However, the maximum lipid yield was 0.56 g L^{-1} equating to 17 mg g^{-1} wheat straw, which is considerably less efficient than other reported values. Arabitol and 2PE were also produced, in lower concentrations than when grown on glucose.

Attempts to improve the lipid yield were unsuccessful, possibly because of too high inhibitor concentrations, not enough sugar and too high nitrogen content. Rapeseed meal was used as a source of nutrients instead of defined chemicals, which increased the cell dry mass, but not the lipid content of the media. Seaweed, particularly when supplemented with rapeseed meal, was a more suitable feedstock than wheat straw, delivering a maximum lipid content of 1.12 g L^{-1}, compared to a maximum of 2.92 g L^{-1} for glycerol.

6 Production of emulsions from lignocellulosic broths by *M. pulcherrima*

6.1 Preamble

One of the key applications of palm oil is as a source of emollients and surfactants used in care products. Care products frequently consist of oil in water emulsions, as emulsions allow for control over rheology, odour and toxicity. The rheological and visual properties are more appealing to consumers than pure oils. Using emulsions, rather than pure oils, also allows the skin to be coated in a thinner film of oil (thus the skin appears less greasy) while using less of the expensive oils by diluting them. The white colours obtained from the scattering of light by oil droplets also have been shown to appeal to consumers.

Recently, due to increasing consumer demand, the care product industry has been increasing the use of natural ingredients. Part of the reason for this is that allergies and skin sensitivities to artificially produced components (colorants, preservatives, stabilisers) have emerged, and consumers believe natural products to be less likely to have these issues.[373] The increasing use of nut oils in emulsions has also led to allergic reactions among a small number of consumers.

While novel components in care products would need to be approved for human use, there is precedent for common oleaginous fungi, such as *Mortierella alpina* and *Mucor*

circinelloides, with no evidence of harm to humans being approved by the US Food and Drug Administration with relative ease for infant formula milk.[196] Yeasts are also widely used in food production including a bread with oleaginous yeasts.[114] It seems likely that *M. pulcherrima,* already used in wine making, would be certified for use in surfactants.

Extraction of oil from oleaginous microbes is costly and usually requires the use of expensive, toxic, non-renewable solvents such as hexane and chloroform.[50, 324]. However, chemical modification of trigylcerides within the yeast cell body, including transesterification of fatty acids within whole yeast cells using microwave radiation, has been demonstrated,[355, 374] indicating that the chemical modifications necessary to generate the range of compounds required for a care product emulsion could potentially take place without solvent extraction.

Prior work has also demonstrated that the carbohydrate β-glucan that comprises 55-65% of *S. cerevisiae* cell walls, once extracted and purified, can be used to stabilise oil in water emulsions of mayonnaise.[375] It is also known to act as a thickener, forming hydrogels that increase the viscosity of the aqueous phase, and thus stabilising the emulsions by increasing the amount of energy needed for oil droplets to move through the aqueous phase.[376, 377] As well as the carbohydrate present in the yeast cells, the fermentation broth is known to contain oligosaccharides, probably glucans and xylans. Glucans are used to alter the rheological properties and improve the stability of oil in water care product emulsions, functioning in the same way as β-glucans from yeast, as well as surrounding oil droplets to provide steric hindrance to coalescence. They are not surface active, and are instead highly hydrophilic and so stabilise the emulsion by depletion stabilisation, in which close approach of droplets results in ever decreasing concentrations of glucan between the droplets which continues until further movement becomes thermodynamically disfavoured.[378]

Proteins are also known to stabilise colloids, such as bubbles of CO_2 in bread dough being stabilised by gluten, and oil in water emulsions being stabilised by proteins in milk.[379, 380] Proteins act as emulsion stabilisers by adsorption at the oil-water interface, they denature and form gel-like networks around the oil droplet, and in so doing sterically stabilise them. This also lowers the surface tension, encouraging emulsification to occur. Providing the pH of the solution is lower or higher than the isoelectric point of the protein, the amphoteric nature of the proteins means they also add a positively charged surface to the oil droplet, stabilising the emulsion by causing electrostatic repulsion between oil droplets. This can sometimes be enhanced by an electric double layer of ions in the

aqueous solution that forms around the charged sphere. The isoelectric point of proteins that are not coordinated to metal is around pH 4.1-5.2.[21, 381]

In order for the oil in water emulsion to be stabilised, the surfactant or protein must surround the oil droplet before the droplet can collide with another droplet and coalesce, making the kinetics of droplet formation and diffusion to the interface important in forming stable emulsions.[379] Due to the presence of lipids, proteins, and suitable carbohydrates as well as 2-phenylethanol, in the yeast and the fermentation broth, the feasibility of using the whole yeast cell along with the supernatant as the sole components in a care product emulsion was investigated.

6.2 Composition of emulsion components

The components available from the *M. pulcherrima* cell residues are given in Table 6.1

Feedstock/component	Lipid	Protein	Carbohydrate	Ash
Glucose	25%	25.22 %	28.8 %	21 %
Wheat straw	12%	41.6 %	7.9 %	39 %

Table 6.1. Components of *M. pulcherrima* grown on glucose or wheat straw

As the lipid is present intracellularly the cell wall must be broken first. Triglycerides can be converted into emulsifiers, surfactants with long hydrophobic moieties and shorter hydrophilic groups that stabilise emulsions. Such compounds can include monovalent fatty acid salts (soaps), zinc, sodium and magnesium ions.

The protein content of the yeast cultured on glucose is lower than that grown on wheat straw, corresponding to the higher lipid content. The ash content of the *M. pulcherrima* grown on glucose is relatively high compared to similar yeasts,[375] and higher still when cultured on wheat straw. This is potentially due to the elevated levels of minerals in the medium from the fertiliser or soil. *M. pulcherrima* is known to scavenge iron from the broth,[318, 319] and this may mean the iron content of the cells is higher. ICP-MS analysis of the cells would confirm this.

Due to the high volumes needed, a model for *M. pulcherrima* oil was required for the preliminary emulsion formation experiments. The FAME profiles of *M. pulcherrima* oil were determined and compared to literature data of rapeseed oil and palm oil. *M. pulcherrima* cultured on glucose produces an oil with a similar lipid profile to palm olein

whereas *M. pulcherrima* cultured on wheat straw produced an oil more similar to palm stearin (Figure 6.2). The lipids from *M. pulcherrima* cultured on glucose and palm olein mostly consist of oleic acid (18:1), followed by palmitic acid (16:0), then linoleic acid (18:2), though the *M. pulcherrima* lipid contains less palmitic acid and more oleic acid, as well as a different composition of other fatty acids present in lower quantities. Palm olein is approximately 45% saturated, and *M. pulcherrima* oil grown on glucose is 26%, which explains why this sample of *M. pulcherrima* oil was observed to be less viscous at room temperature than palm oil. This could be impactful on the final product as even small differences in fatty acid composition can mean the replacement is unsuitable.[196] On the other hand, the wheat straw cultivated sample had proportions of oleic and palmitic acid within three percent of those of palm stearin albeit with a far simpler composition consisting of only four fatty acids. The high proportion of oleic acid and undetectable levels of minor fatty acids suggests that the elongating and desaturation enzymes are not functioning or absent when *M. pulcherrima* is grown on wheat straw. Palm stearin is around 63% saturated, *M. pulcherrima* oil cultivated on straw is around 60% saturated, which is probably the viscosity is similar.

The surface tension (oil/air) of the lipid produced from *M. pulcherrima* was analysed by pendant drop tensiometry, and compared to both palm and rapeseed oil (measured with Du Nouy ring, (see 2.5.2 and Figure 6.3.)

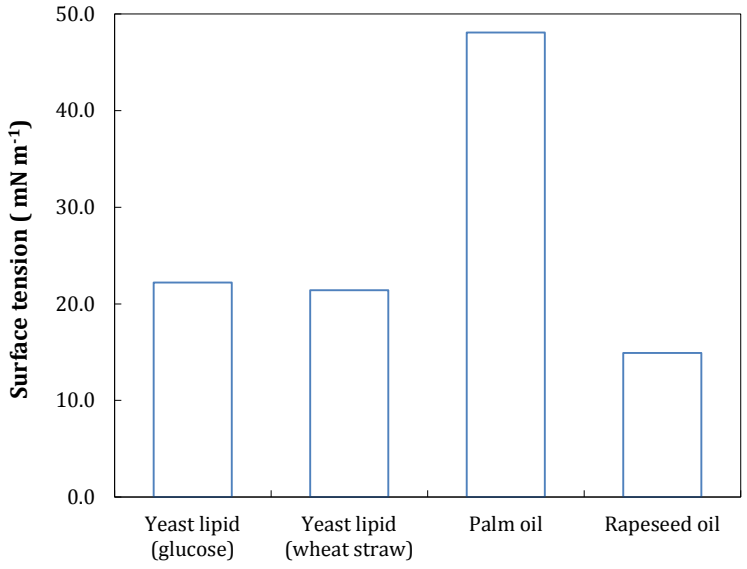
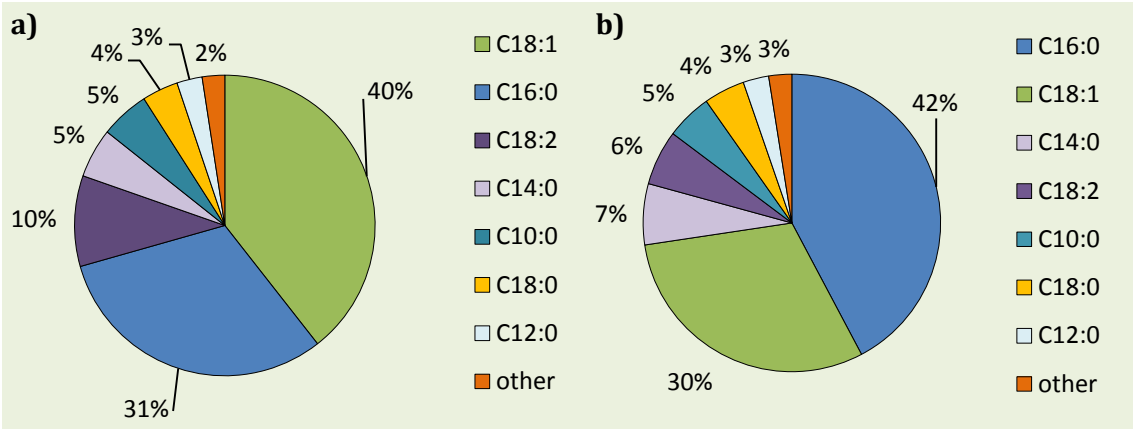
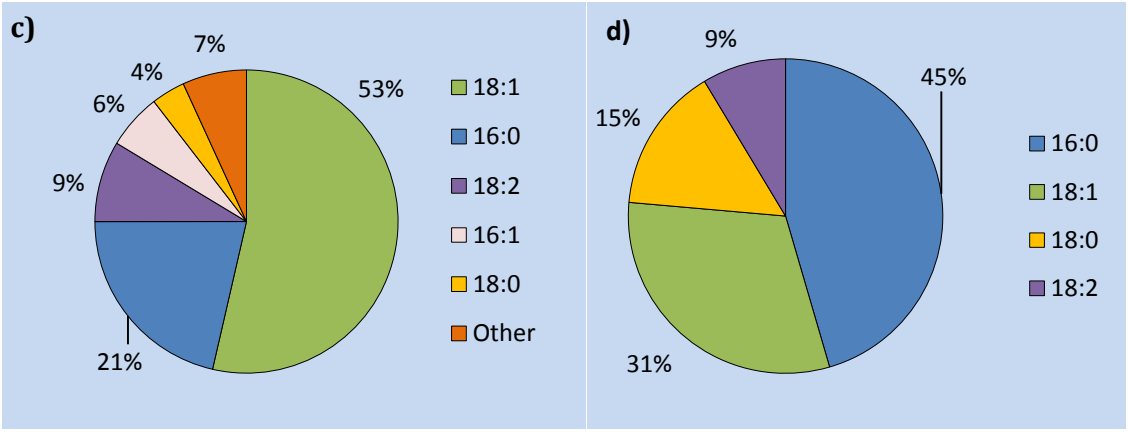


Figure 6.1. Surface tension (oil/air) of yeast (*M. pulcherrima*) lipid, measured by tension drop tensiometry. Surface tensions of palm oil and rapeseed oil were measured by Du Nuoy ring.

Palm oil



***M. pulcherrima* oil**



Rapeseed oil

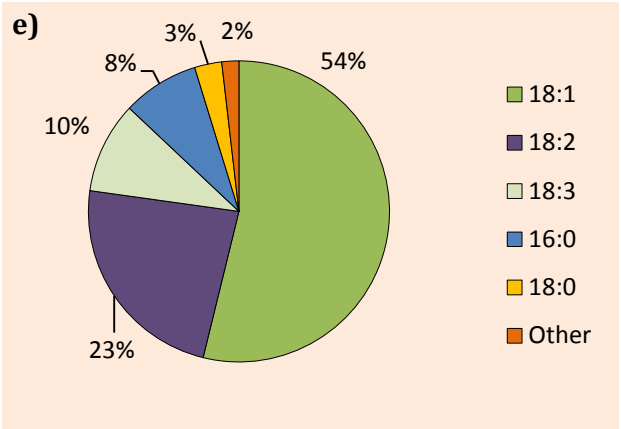


Figure 6.2. Composition by % of fatty acids in the two main fractions of palm oil – a) palm olein[17] and b) stearin, [17] the oils produced by *M. pulcherrima* when grown on c) glucose and d) wheat straw as well as the FAME profile of e) rapeseed oil.

M. pulcherrima oil grown on glucose (22.2 ±0.05 mN m^{-1}) was comparable to that of *M. pulcherrima* oil grown on wheat straw (21.4 ±0.1 mN m^{-1}). These values are intermediate between rapeseed oil (14.9 mN m^{-1}) and palm oil (48.1 ±4.2 mN m^{-1}), but more similar to rapeseed oil. While a value was obtained for palm oil, the oil was too viscous at room temperature to allow the ring to penetrate the interface properly, typical literature values for palm oil at 40 °C put the value at 31.5 mN m^{-1}, more similar to the *M. pulcherrima* oil.[382] Literature values for rapeseed oil at 20 °C are 33.8 mN m^{-1}.[382] *M. pulcherrima* lipid having a lower surface tension than the literature values of rapeseed oil could help it to form oil droplets.

Constituent	Concentration (g L^{-1})
Oligosaccharides	Unknown
Xylose	0.01
Acetic acid	0.06
Arabitol	0.016

Table 6.2. Constituents of fermentation broth after *M. pulcherrima* was grown on liquid hot water pretreated wheat straw (batch process, 120 °C, 60 min), no supplemental nutrients, 15 days (three at 25 °C twelve at 15 °C), 180RPM, yeast removed by centrifugation and autoclaved.

The fermentation broth consisted primarily of oligosaccharides, comprising 69% of the total area under the HPLC trace, although the concentration in g L^{-1} is unknown (Table 6.2). The only monosaccharide detectable was xylose, and acetic acid was the only acid, in very small quantities. The high quantity of oligosaccharides was expected to provide viscosity-improving qualities as on visual inspection, the broth produced foam when shaken.

6.3 Surface-active properties of *M. pulcherrima* residue and fermentation broth

The feasibility of using the products of *M. pulcherrima* grown on wheat straw to stabilise an oil in water emulsion was examined by investigating if the fermentation broth and the yeast itself had surfactant properties. The concentration of surfactants at the interface is higher than in the bulk solution as this allows the free energy to be increased and lowers the surface tension. The ability for compounds to act as surfactants can thus be measured by adding them to a solution and measuring any change in surface tension.

Therefore, yeast and broth were each added to water at increasing concentrations and the air/water surface tension was measured. This was followed by adding rapeseed oil to the top of the water and measuring the oil/water interfacial tension in order to see if the components could interact with the oil/water interface, which is more important in forming oil droplets. (Figure 6.3a and b). Due to the number of experiments required and the high viscosity of palm oil, rapeseed oil was used to determine the reduction in the oil-water surface tension. Combinations of yeast and broth were also tested, to see if there were any positive or negative interactions between these components (Figure 6.3c).

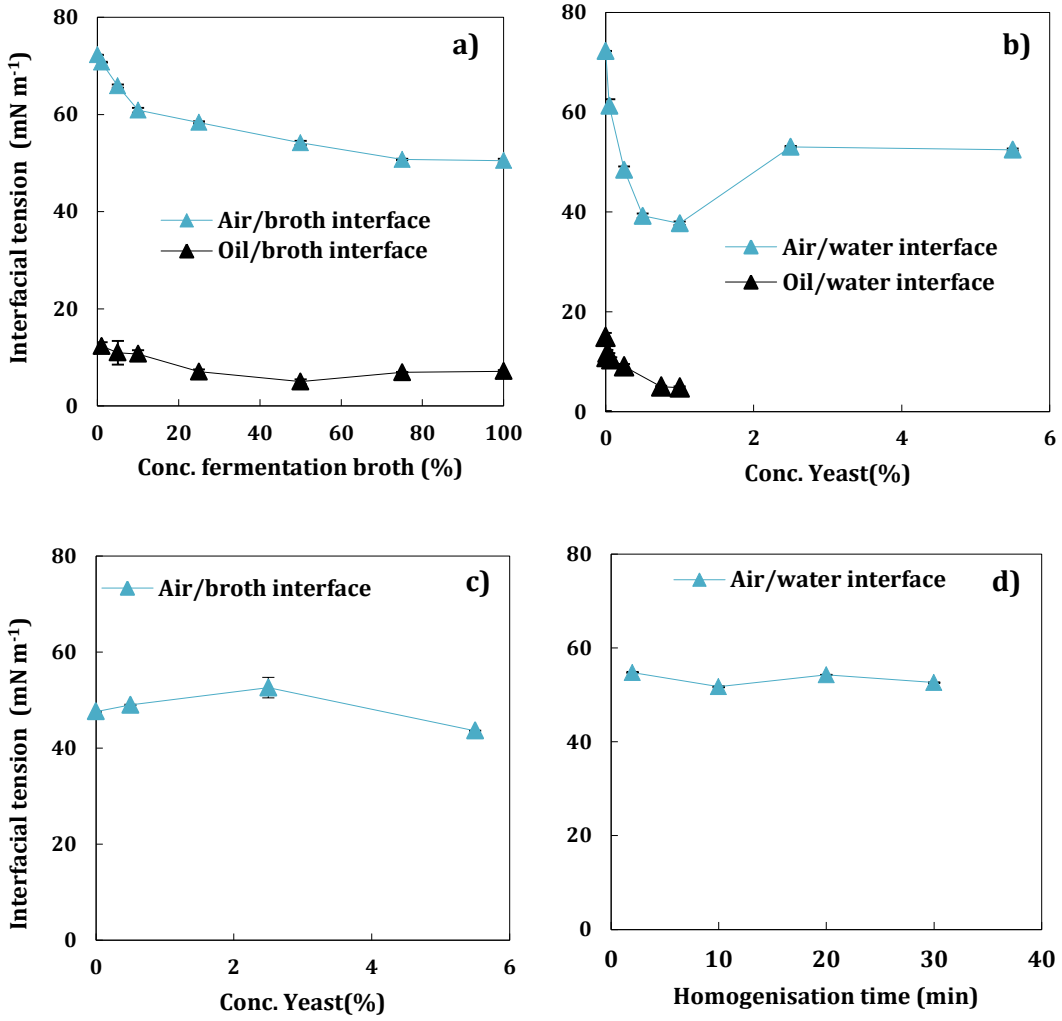


Figure 6.3. Effect of fermentation products on interfacial tensions. Air/aqueous phase and oil/aqueous phase surface tensions with a) increasing concentrations of fermentation broth, b) increasing concentrations of yeast residue in water, c) 100% broth with increasing concentrations of yeast residue, d) water with yeast residues homogenised for increasing amounts of time.

To avoid interference of *M. pulcherrima* lipid being a factor in the results and to allow for control of the ratio of oil to yeast and broth, oil was extracted from *M. pulcherrima* (as per section 2.5.1) and the remaining yeast residue used. Water was also removed in order to standardise the amount of yeast added. To ensure the yeast was well dispersed in the water, it was first homogenised for two minutes. To investigate whether homogenisation led to a change in the surfactant capabilities of the yeast (e.g. by denaturing the proteins), the measurements were repeated on the same sample homogenised for increasing amounts of time, up to 30 minutes (Figure 6.3d).

As the concentration of fermentation broth increases, the air/broth interfacial tension decreases, with the greatest effect of the broth concentration on the surface tension being from between 0 and 20 % (Figure 6.3a). At broth concentrations greater than 20%, the surface tension decreases less rapidly. This suggests that some components in the broth are surface active, and probably move to the surface and disrupt the hydrogen bonding between the water molecules. The oligosaccharides are unlikely to be surface active unless they have hydrophobic groups such as lipids or proteins bound to them,[378] it is possible that the surface active components are 2-phenylethanol or organic acids. The oil/broth interfacial tension was similarly decreased by increasing concentrations of broth, with a minimum tension at 50% broth concentration.

Addition of yeast residue also led to a reduction in air/water interfacial tension, with a minimum at 1% wt loading of yeast, the yeast residue has a greater effect on the interfacial tension (37.7 mN m^{-1}) than the products in the fermentation broth (51.5 mN m^{-1}), although the minima were similar for the oil/aqueous interface (4.8 vs 5.0 mN m^{-1} respectively). This demonstrates that while there are presumably some form of extracellular polysaccharide in the broth, they do not affect the properties significantly at the bulk level. The air/water interfacial tension increases with concentration of yeast residue over 1 wt%. This suggests a yeast-yeast interaction is occurring, possibly due to coagulation of proteins and carbohydrates. Another mechanism for oil droplet stabilisation by the yeast residue is for finely divided particles to be preferentially wetted by water, and move to the interface.[21] These particles may reach the surface faster than the proteins, yet be less effective at lowering the surface tension.[379] Alternatively, there may be a difference in charge between the carbohydrates and the proteins, as such complex patterns in surface tension with increasing concentrations of surfactant are seen when polyelectrolytes and surfactants interact. The water the yeast residue is suspended in is at pH 7, which is likely to be above the isoelectric point so the proteins will be

negatively charged.[383] Potentially yeast DNA, a polyelectrolyte, also interacts with the mixture.[383, 384] However, given the quantities of yeast present, this seems unlikely. Commercial surfactants lower the air/water surface tension to around 35 mN m^{-1}, which is only slightly lower than the yeast residue attained.

To assess the effect of both these factors on the surface tension the yeast residue was added to the fermentation broth, as opposed to the water used previously. Interestingly the yeast residue does not significantly lower the surface tension of the system (Figure 6.3c). Adding yeast to the fermentation broth initially appears to increase the surface tension slightly, then at higher yeast concentrations it decreases, which may indicate an interaction between the components of the yeast and the broth, such as the proteins and oligosaccharides coagulating.[384] Homogenisation time did not affect the surface tension, indicating that even if it did denature proteins, the denatured proteins were as effective at lowering the surface tension as the non-denatured proteins (Figure 6.3d).

6.4 Emulsion formation and stability testing

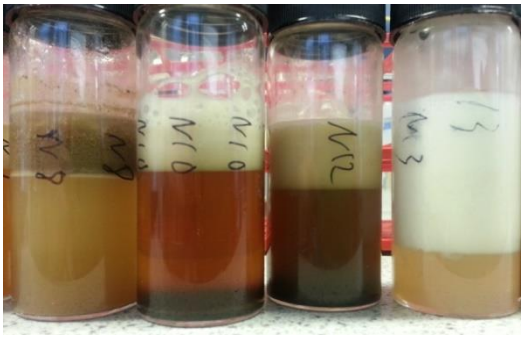
In order to examine the ability of yeast residue and broth to stablise an oil in water emulsion for use in care products, a series of emulsions were formulated with varying quantities of yeast residue, rapeseed oil, saponified rapeseed oil and concentrations of fermentation broth (Table 6.3.) The components and ranges were chosen for the following reasons. Whilst the yeast residue and fermentation broth did not reduce the surface tension as much as commercial surfactants, the *M. pulcherrima* oil could be made into surfactants, such as soap. The maximum reduction in surface tension with the yeast residue alone was attained with 1 wt% loading, however to prevent sedimentation, the concentration was limited to 0.5 wt%. The amount of oil in cosmetic products varies from less than 1 wt% in shampoos to around 20 wt% for creams, and so the quantity of rapeseed oil in this study was varied between these two points.[373, 385] The surfactant portion typically varies from 5 wt% in creams to 40 wt% in shampoos.[373, 385] The soap fraction was therefore varied between these levels. Two common preservatives, 2-phenoxyethanol (1 wt%) and EDTA (0.1 wt%) were also added. EDTA was used as it chelates metal ions and so prevents the ions from catalysing the oxidation of unsaturated fatty acids.[380] The antimicrobial 2-phenoxyethanol was chosen as it is thought to be less harmful to humans than parabens.[386]

Entry	Oil [% wt]	Soap [% wt]	Yeast residue [% wt]	Broth [%]	Relative CI W 0	Relative CI W 1	Relative CI W 2	Relative CI W 3	Relative CI W 4	Relative CI W 8
1	1	0	0	0	-3.49	-2.14	2.08	3.61	0.68	0.49
2	1	0	0.5	0	-4.80	-3.65	-2.19	-2.96	-5.36	-3.11
3	1	0	0	100	-6.63	-6.86	-7.00	-7.55	-7.35	-5.17
4	1	0	0.5	100	-4.48	-6.10	-6.47	-5.93	-5.22	-2.77
5	1	5	0.25	50	9.12	8.63	7.31	6.92	3.79	5.38
6	1	10	0	0	12.71	4.20	4.14	5.37	2.92	4.42
7	1	10	0.5	0	nd	16.41	14.94	11.29	12.79	13.37
8	1	10	0	100	41.06	31.23	18.28	20.68	13.23	-4.91
9	1	10	0.5	100	31.46	21.82	20.23	16.04	13.31	13.90
10	10.5	0	0.25	50	-2.30	-5.16	-3.53	-3.69	-4.36	-4.97
11	10.5	5	0	50	11.88	9.51	8.92	7.42	7.46	6.24
12	10.5	5	0.5	50	11.16	4.84	4.89	3.23	4.10	-9.13
13	10.5	5	0.25	0	4.02	5.54	4.22	4.66	3.16	4.65
14	10.5	5	0.25	100	3.05	2.61	2.68	5.00	-8.86	0.12
15	10.5	5	0.25	50	6.41	7.48	7.83	5.72	6.52	5.99
16	10.5	5	0.25	50	8.94	3.42	2.68	3.97	4.92	4.12
17	10.5	5	0.25	50	5.18	2.82	12.44	0.93	0.35	1.37
18	10.5	10	0.25	50	23.32	13.94	12.93	9.79	8.82	7.70
19	20	0	0	0	-6.61	-3.99	-7.65	-2.36	-2.57	-2.66
20	20	0	0.5	0	15.80	7.22	5.57	5.40	4.53	5.33
21	20	0	0	100	0.13	-1.33	-2.74	-1.55	-0.89	-0.83
22	20	0	0.5	100	2.19	3.58	3.62	3.43	3.95	3.03
23	20	5	0.25	50	14.06	13.15	8.32	8.34	7.51	8.30
24	20	10	0	0	46.49	22.62	20.94	nd	20.99	4.36
25	20	10	0.5	0	40.28	37.41	32.09	35.74	33.43	19.45
26	20	10	0	100	22.39	29.92	26.01	26.15	25.78	22.32
27	20	10	0.5	100	37.07	34.02	30.84	17.85	28.16	28.67

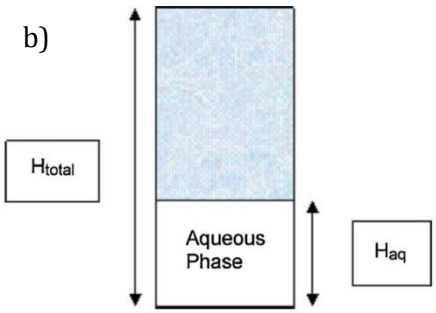
Table 6.3. Emulsion composition and relative creaming index (CI, Figure 6.4) over eight time points. All emulsions included preservatives 0.1% EDTA and 1% 2-phenoxyethanol then homogenised. Experiments chosen via a central composite face design.

The pH of protein stabilised emulsions and particularly care product emulsions is extremely important, this is because proteins are better able to stabilise colloids if they are charged, which means the pH should not be at the isoelectric point. Interfacial tension decreases and interfacial elasticity, an important but poorly understood property that appears to correlate with emulsion stability and small droplet size, increases with increasing pH.[381] However, the pH of the skin is slightly less than 5 for men and slightly higher than 5 for women,[387] the cosmetic formulation should not alter this,[388] therefore for this study a pH of 5 was selected. Emulsions were prepared according to a response surface methodology, with a central composite face design, quadratic model, using the statistical package MODDE (Table 6.3.)

a)



b)



c) $$CI = \frac{H_{aq}}{H_{tot}} \times 100$$

d) $$Relative\ CI = \frac{CI_{initial}}{CI_{final}} \times 100$$

Figure 6.4. a) Representative examples of emulsions formed showing cream phase (upper) and aqueous phase (lower) as well as sedimentation. From left to right – table 6.3 entries 22, 7, 9 and 24; b) Creaming index (CI) consisting of total height (H_{total}) and aqueous layer height (H_{aq}). Reprinted from C.D. Ampatzidis, E.-M.A. Varka and T.D. Karapantsios,[389] Copyright 2014, with permission from Elsevier; c) equation for creaming index; d) equation for relative creaming index.

Stability of the emulsion is crucial. Care product emulsions are almost always found as a single phase, and so creaming is undesirable. Creaming is the separation of the emulsion into two layers due to different densities of the oil and aqueous phases, and although the top layer is still emulsified, it makes coagulation and subsequent coalescence more likely.[21] The rate and extent of creaming over time is a measure of emulsion stability. The creaming index (CI, Figure 6.4) is a method of quantifying this, with a perfectly homogenous emulsion having a CI of 100. As different volumes of oil were used, the minimum creaming index that can be achieved varies and so the relative creaming index was developed in order to compare the CI to the minimum CI (oil on water) allowing all samples to be compared with one another.

The emulsions were kept stationary at room temperature and the relative CI was measured on a weekly basis for 4 weeks, and at 8 weeks in order to examine the extent of separation over time. Models for each time point were then defined by examining the correlation between all factors (and interactions) and the responses. Factors that were insignificant, defined by those whose removal led to the increase in the Q2 value (a measure of the predictive power of the model), were removed.

In order to examine the accuracy of the models in predicting the responses, the values predicted for each of the scenarios in Table 6.1 were compared to the observed values for these scenarios (Figure 6.5). If the model were perfect, the predicted values and observed values would be identical, so the correlation between these values would be linear and highly significant. The null hypothesis that there is no correlation between the observed and predicted values was analysed by ANOVA.

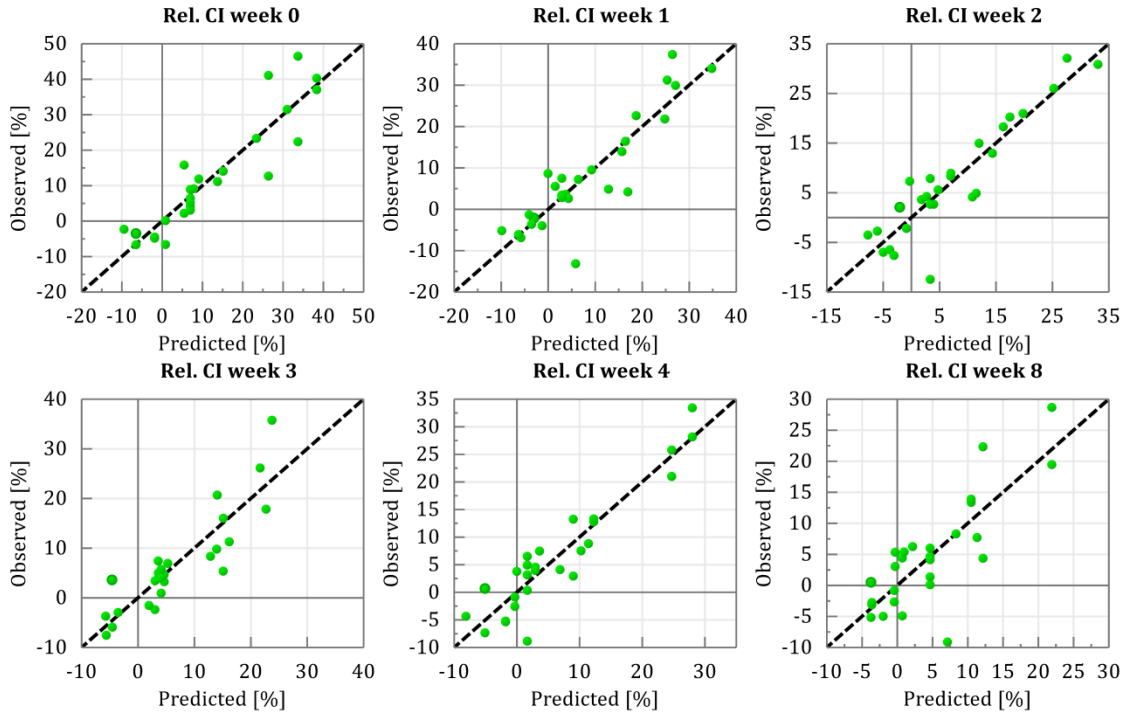


Figure 6.5. Correlations between predicted values and observed values for relative CI at each time point. N=Number of value; DF = degrees of freedom; R2=R^2.

Time point (weeks)	F	p
0	21.1209	0.000
1	10.9758	0.000
2	11.9283	0.000
3	14.1282	0.000
4	22.5033	0.000
8	7.81824	0.000

Table 6.4 Results of ANOVA analysis on the observed vs. predicted.

The models described the experimental data significantly better than chance, as shown by the F and p values. The reason for the unexplained variance may be due to the imprecision of the lipid content data (Appendix 3). Once the models were determined, the response surfaces predicted by the models indicate the most important factors and the direction the design space should be moved towards, if unconstrained by techno-economic reasons.

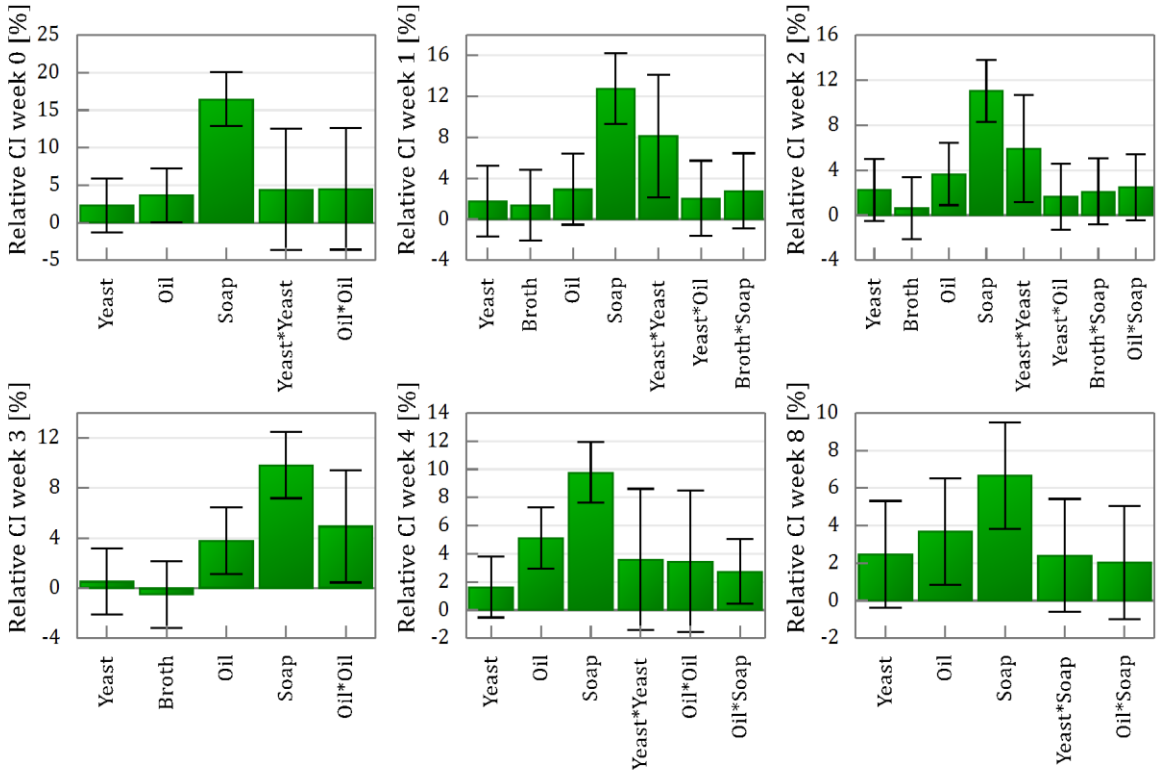


Figure 6.6. Models for stability of emulsions formed from *M. pulcherrima* fermentation products. Stability measured in terms of relative creaming index (CI). Figure shows the factors (and interactions between factors) that contribute to the relative creaming index for each time point. Coefficients with two terms multiplied (*) together indicate an interaction between the two factors.

The models used to fit the data included different coefficients at each time point. At week 0 (24 hours after homogenisation), the most stable emulsions were those with 1 g L^{-1} yeast, 20% wt oil and 10% wt soap – the broth having no effect. The creaming index after 24 hours was not significantly affected by the amount of yeast residue alone, however the interaction between yeast and itself (yeast*yeast) was larger. This results in the curvature seen in the response surfaces (Figure 6.8, 6.9 and 6.10) where the emulsions are made less stable by the addition of yeast up to a minimum at ~0.2 g L^{-1} yeast, then the stability increases with yeast concentration from this point onwards. This could be because the proteins adsorb at the interface, lowering the interfacial tension between the oil and the water allowing smaller droplets to form, but the surface tension is not lowered enough, nor is the steric stabilisation sufficient to prevent creaming. However, there is a larger

surface area for more proteins and carbohydrates to adsorb onto, so when more yeast is added a second layer of protein could form which helps to sterically stabilise the droplets.

Conformational changes of the proteins are also known to occur in protein stabilised oil droplets. Higher concentrations may lead to more stable conformations, and lower surface tension as concentration of emulsifiers is well known to lower the surface tension and decrease the droplet size.[379, 390] The other way that the yeast could be stabilising the oil droplets is by the presence of particles, as per a Pickering emulsion. The competition for the interface between the particles, proteins and carbohydrates (in emulsions where no soap is present) may account for the shape of the response surfaces.

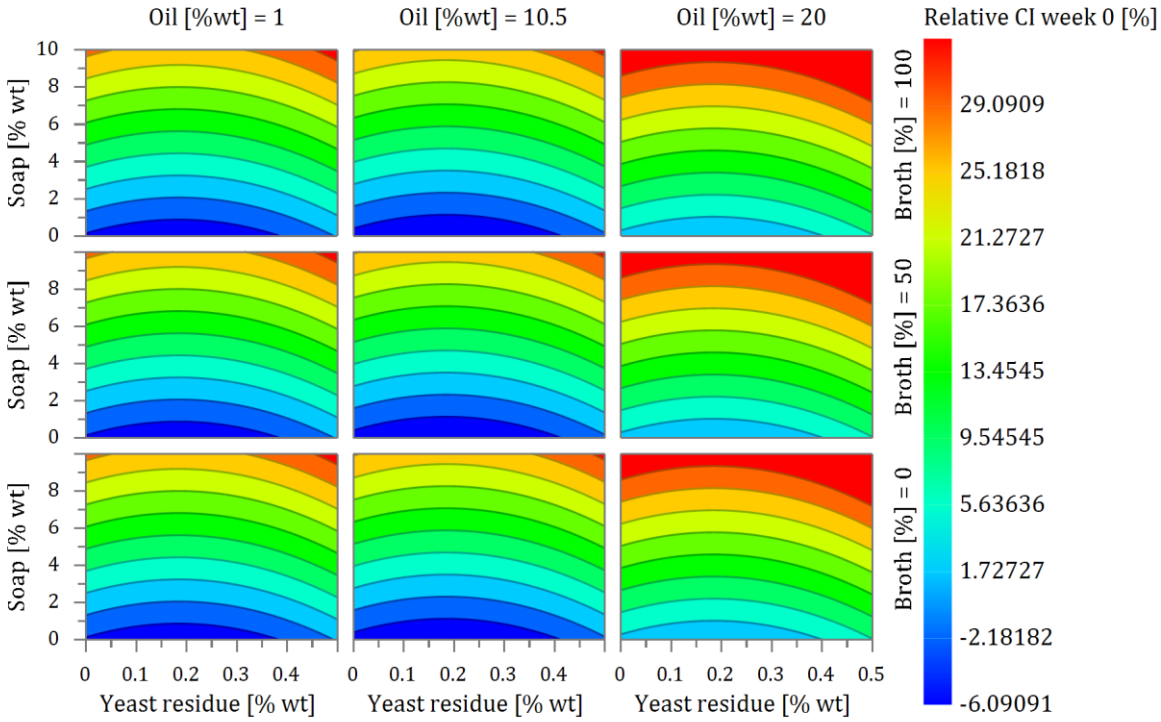
The amount of oil was an important factor, with greater quantities being apparently more stable, as well as there being a significant oil*oil interaction. This is probably because the emulsions are kinetically stabilised and if the rate of coalescence were the same it would take longer for the larger volume of oil to coalesce. Also, the loss of oil from the system due to sticking to the sides of the vessel is less significant in the case of larger oil volumes. The total volume of the emulsions is greater than the total volume of the unemulsified components (oil on water), and the total volume of the cream layer is larger than the volume of unemulsified oil. This is because it consists of a matrix of oil in water, with the cream layer's volume further expanded by the repulsions between the water and oil molecules, and repulsions between the surfactants, proteins and carbohydrates. The more oil that is present, the greater the increase in volume will be. This hypothesis was borne out in the data, with a reduction in total volume between week 0 and week 8 of 0.4%, 3.7%, 11%, for oil at 1%, 10.5% and 20% wt respectively.

Unsurprisingly, soap concentration had the largest effect on emulsion stability, as this is a more ideal surfactant capable of lowering the interfacial tension and thus the thermodynamic instability of the emulsion. It is highly likely that there is competition for the interface between the proteins, soap and yeast particles. If the yeast proteins move to the interface faster than the soap, but are less stabilising, perhaps due to being at the wrong pH, the stability may be reduced. It seems likely that additional surfactants, such as soap, would be necessary to create a stable emulsion, although the amounts of soap added in this study were not sufficient to create an emulsion stable for as long as 24 hours. Polymerisation of the oil with polyethylene glycol could make more effective surfactants.

The relative CI reduced in all emulsions with time. As the emulsions aged, different factors (and interactions between factors) became too important to remove from the model without reducing its predictive power. Despite this, weeks 0, 1, 2 and 4 have similar response surfaces, only at decreasing relative CIs (Figure 6.8, 6.9 and 6.10). Out of all the factors the broth had the least significant effect on the creaming index, including having a slightly negative effect on the week 3 creaming index. This indicates that the surfactants within it were less effective than the soap and yeast residue at lowering the surface tension, which is the opposite of what was found in the surface tension measurements. This suggests that the compounds that are effective in a simpler system of oil and broth are becoming inactivated, perhaps by binding to the other components or, in the case of 2-phenylethanol, evaporating. Alternatively, they may not reach the surface of the oil droplets, as the hydrophilic hydroxyl groups on 2-PE and acids prefer the aqueous phase, and so do not successfully compete for the surface of the oil droplets. It was hypothesised that the oligosaccharides would behave as β-glucan does in oil in water emulsions, stabilising the emulsions by thickening the aqueous phase and by depletion flocculation. These mechanisms do not appear to have been effective as perhaps the concentrations were too low.

In weeks 1 and 2 there are yeast-oil interactions, and in week 8 a yeast–soap interaction leading to the distinctly different shape of the response surface. There are significant oil-soap interactions in weeks 2, 4 and 8. This suggests that both yeast and soap are at the interface between the oil and the aqueous phase and that this helps to stabilise the oil droplets. There were some interactions between the soap and the other surface active compounds, however these were not always significant at each time point. There was no significant yeast-broth interaction, suggesting that the oligosaccharides in the broth did not associate with the yeast residue, or if they did, this did not affect the emulsion stability.

a)



b)

Figure 6.7. a) Predicted response surfaces for the emulsions 24 hours after emulsification (week 0) using varying quantities of soap, yeast residue, and oil, and fermentation broth. The least separated emulsions were made using the compositions in the red areas. b) Images of emulsions immediately after homogenisation. Compositions from left to right are Table 6.3 entries 26, 15, 8 and 20.

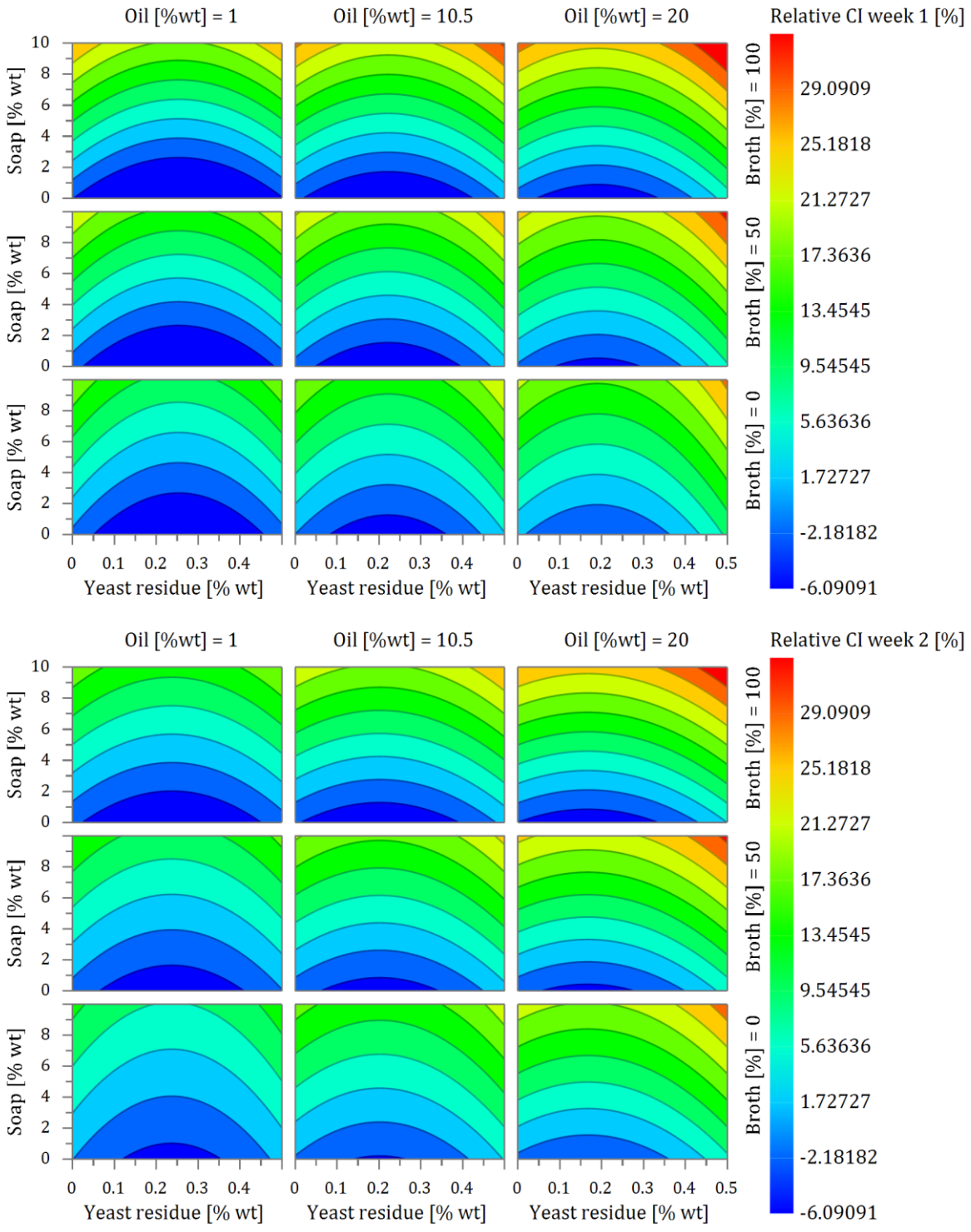


Figure 6.8. Predicted response surfaces for the emulsions at week 1 (upper) and week 2 (lower) using varying quantities of soap, yeast residue, and oil, and fermentation broth.

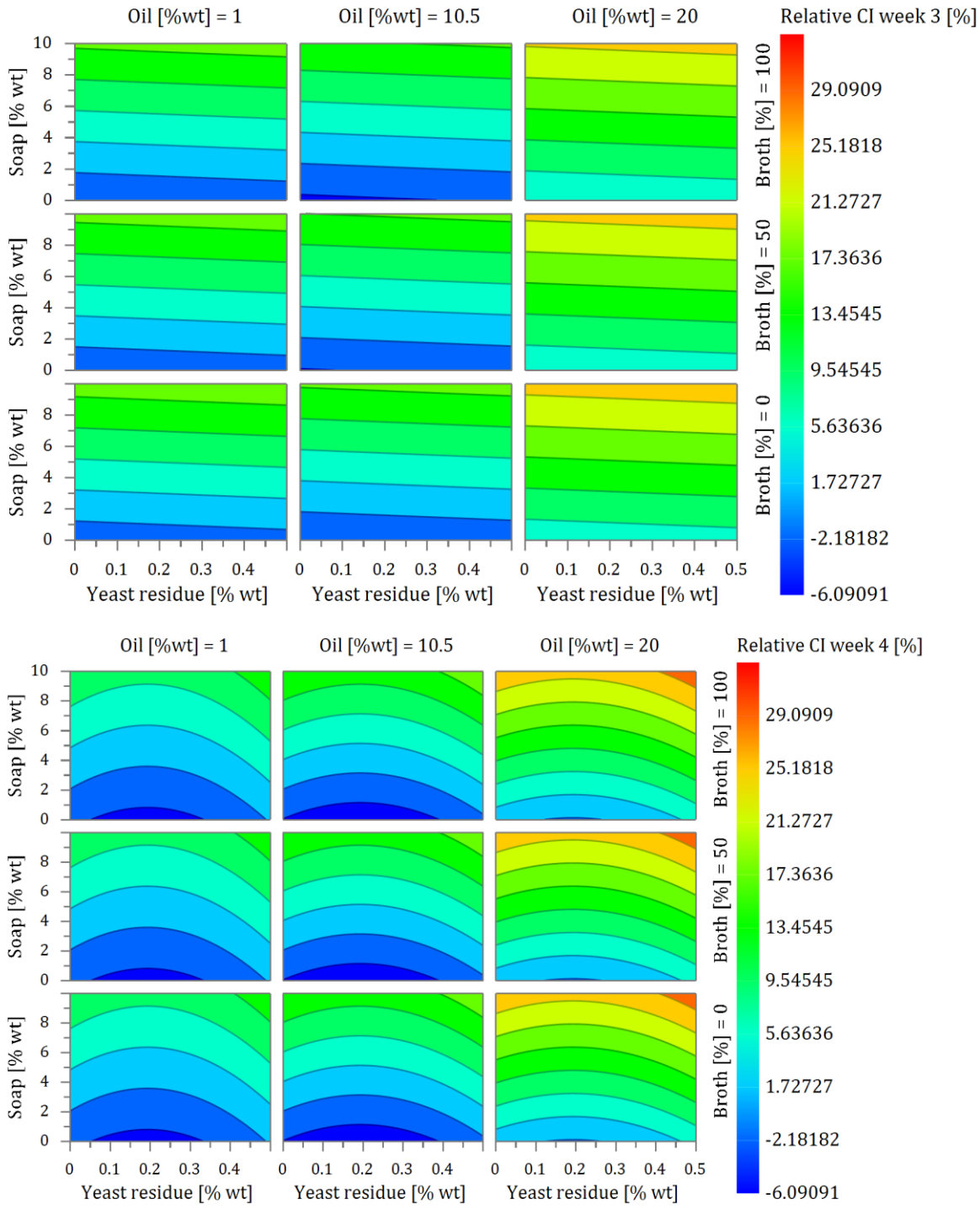


Figure 6.9. Predicted response surfaces for the emulsions at week 3 (upper) and week 4 (lower) using varying quantities of soap, yeast residue, and oil, and fermentation broth.

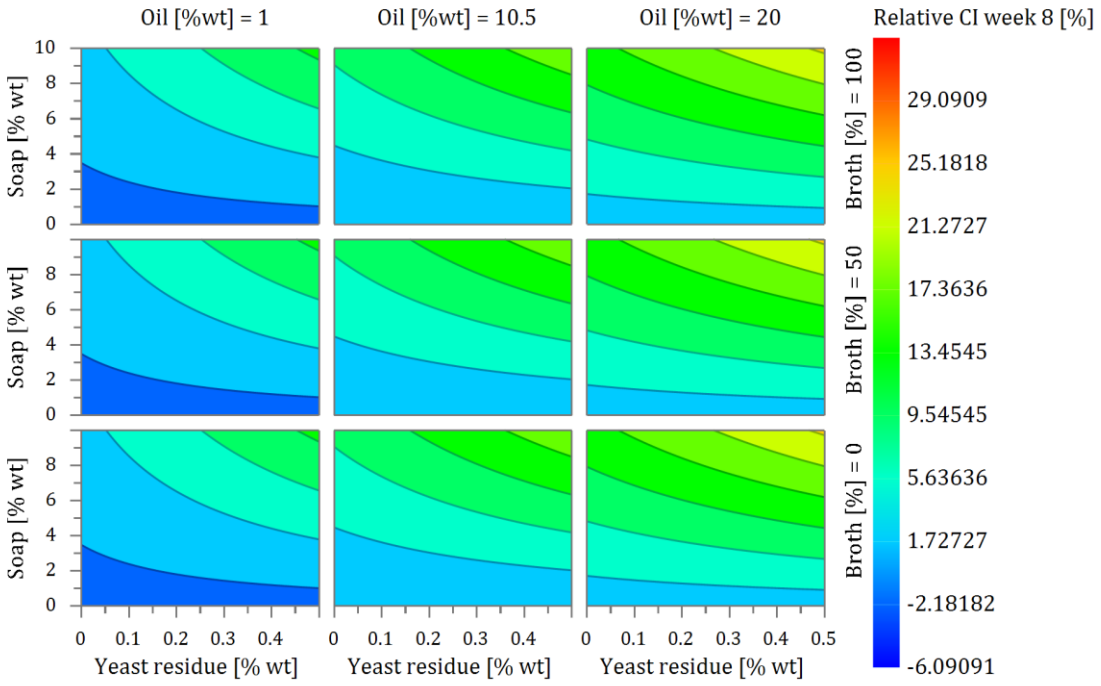


Figure 6.10. Predicted response surfaces for the emulsions after week 8 using varying quantities of soap, yeast residue, and oil, and fermentation broth.

This is a highly complex system with many potential interactions of varying significance. Not all factors are accounted for within the DOE model, such as sodium ion concentration – residual sodium could have come from the soap, or residual sodium hydroxide. The slightly basic soap could have increased the pH of the solution equal to the isoelectric point, making the proteins less stabilising of the oil droplets. At pHs above the isoelectric point the proteins denature and become negatively charged. If the protein is surrounding the oil droplet, the charge is beneficial for stabilising the emulsion as it increases the amount of electrostatic repulsion between the droplets. As the pH of the broth was set to 5 and slightly basic substances were added, the proteins are likely to be negatively charged. In order to establish why the emulsions were unstable, droplet size and distribution was measured.

6.5 Particle sizes

Emulsions consist of droplets of a range of sizes, as there is a dynamic equilibrium between drop coalescence and breakage.[379] A typical emulsion has particle sizes of between 0.1 and 10 μm.[21] Emulsions with a single (monomodal), narrow range (monodisperse) of droplet sizes have a higher surface area to volume ratio so are more stable than those with a wide range (polydisperse) or multiple ranges of droplet sizes (polymodal). This is because larger droplets are more stable, so will cannibalise smaller droplets in order to minimise the total surface energy of the system (Otswold ripening). Monodisperse systems are also sometimes more viscous, an important property in the aesthetics of a care product emulsion.[375] It is therefore important to determine the average size and range of sizes of droplets in order to ascertain how effective emulsification has been, and to investigate the stability of the emulsion by ascertaining the distribution of particle sizes.[379, 391] Dynamic light scattering is widely used for this, and gives the particle size distribution. The measure of particle size that can be determined from this – the Z-average size - is accurate only if the particles are monodisperse. The emulsions were re-emulsified by the same homogenisation method used initially and the particle size distribution immediately afterwards was determined by dynamic light scattering.

All but Table 5.4 entries 6, 14-17 had multiple modes, and so the Z-mean was undeterminable, however most emulsions had particle sizes that ranged between 4-100 μm. Having polymodal distributions explains why the emulsions were unstable, due to the Otswald ripening effect. The reason for the polymodal distribution could have been the homogenisation process as the variation on droplet size is partly determined by the intensity of agitation during emulsification, which promotes both drop coalescence and breakage. The kinetics of adsorption of the emulsifier and the interfacial tension it induces also affect the drop coalescence and breakage, and thus the distribution. There were five emulsions with monomodal distributions (Table 6.5) three of which (15-17) were centre point replicates in the factorial design having intermediate values for oil, soap, yeast and broth.

Label	Mean length (µm)	SD	PDI	Distribution
1	2.5	3.7	2.26	Polymodal
2	4.3	1.9	0.20	Bimodal
3	3.6	2.9	0.65	Bimodal
4	2.3	2.0	0.73	Bimodal
5	3.4	2.2	0.39	Bimodal
6	58.0	12.1	0.83	Monomodal
7	1.6	1.2	0.56	Bimodal
8	2.4	2.0	0.68	Bimodal
9	2.6	1.1	0.18	Bimodal
10	2.1	3.0	2.01	Polymodal
11	2.1	2.0	0.88	Bimodal
12	3.3	2.3	0.48	Bimodal
13	3.1	2.9	0.88	Polymodal
14	2.4	0.0	0.00	Monomodal
15	10.8	4.8	0.20	Monomodal
16	542.8	8.5	0.00	Monomodal
17	530.0	33.8	0.00	Monomodal
18	5.3	15.7	8.61	Polymodal
19	5.3	10.0	3.54	Polymodal
20	5.4	12.6	5.42	Polymodal
21	3.7	4.4	1.42	Bimodal
22	5.6	6.1	1.20	Polymodal
23	2.5	2.3	0.83	Bimodal
24	5.3	3.2	0.37	Bimodal
25	4.0	3.3	0.74	Bimodal
26	4.2	3.2	0.58	Bimodal
27	3.7	2.2	0.37	Bimodal

Table 6.5. Mean length, standard deviation of mean length (SD), polydispersity index (PDI) and number of modes of emulsions.

Emulsions 14, 16 and 17 were also monodisperse, having a PDI less than 0.2. Interestingly, these emulsions were not the most stable in terms of relative CI. Additionally, no correlation was found between polydispersity index and creaming index suggesting that the emulsifiers are not stabilising enough to prevent coalescence. This is supported by the fact that the most stable emulsions with relative CIs over 5 had either 5% or 10 % soap.

The majority (15/27) of the emulsions showed a bimodal distribution with a size of approximately 3 ±2.5 µm. Many of these were also the most stable emulsions. The effect of the emulsifying agents on the particle size distribution of emulsions with 1% oil is shown in (Figure 6.11).

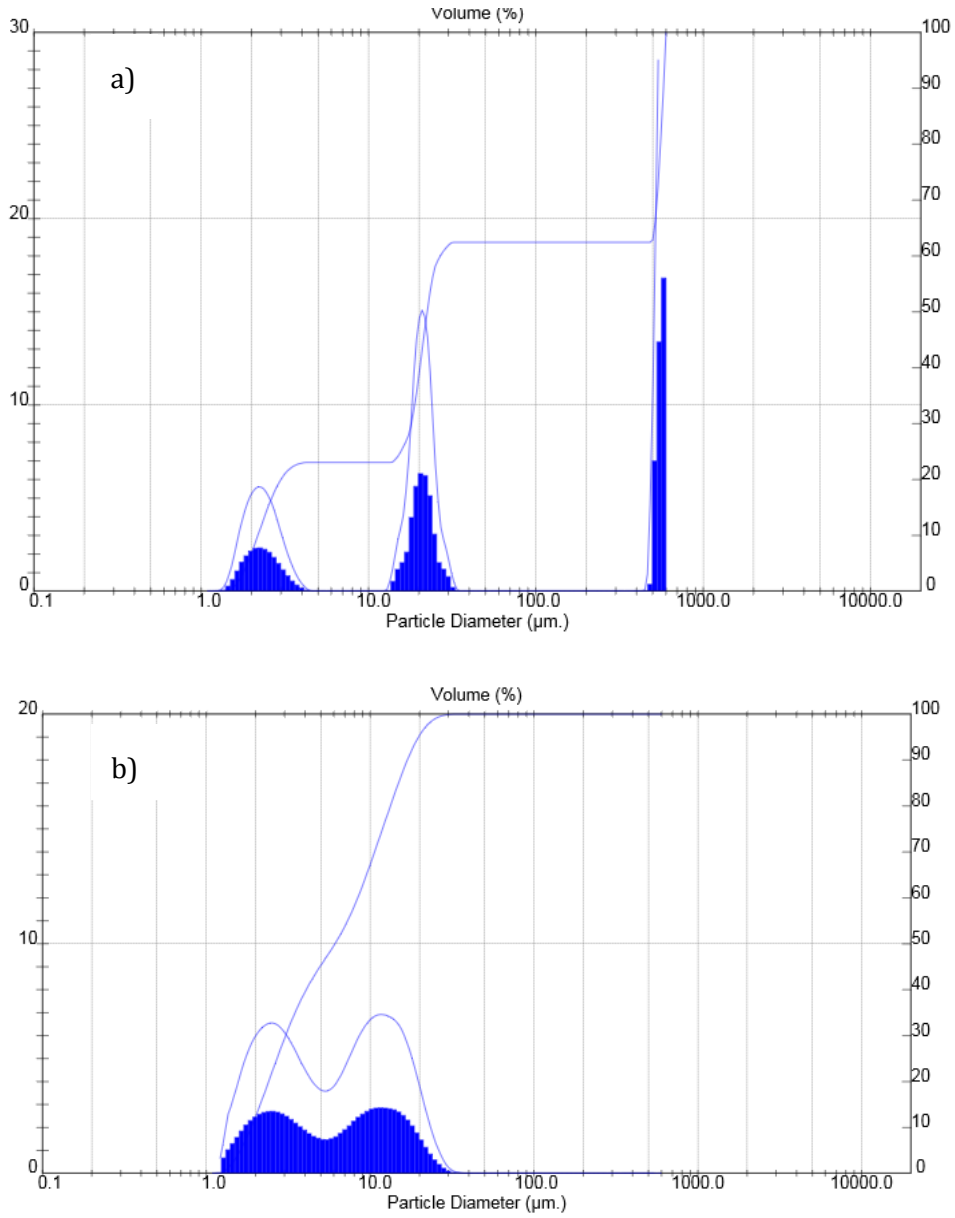


Figure 6.11. Size distribution of a) emulsion 1 with 1% oil and no other components except preservatives immediately after homogenisation. There were three modes after homogenisation at 1.32-4.30 µm, 11-37 µm, 492-600 µm with proportions of particles in these modes at a ratio of 1:1.6:1.7 ratio b) emulsion 8 with 1% oil, 100% broth and 10% soap with more dispersed modes centre around 2 and 12 nm at approximately equal proportions.

Immediately after homogenisation of the emulsion containing only oil, there are three modes, centred round 1.5 µm, 11 µm and 600 µm. When soap and broth are added, the size distribution become more dispersed and smaller in size. Once particles coalesce to sizes over 1000 µm they are no longer detectable using this DLS system and lens.

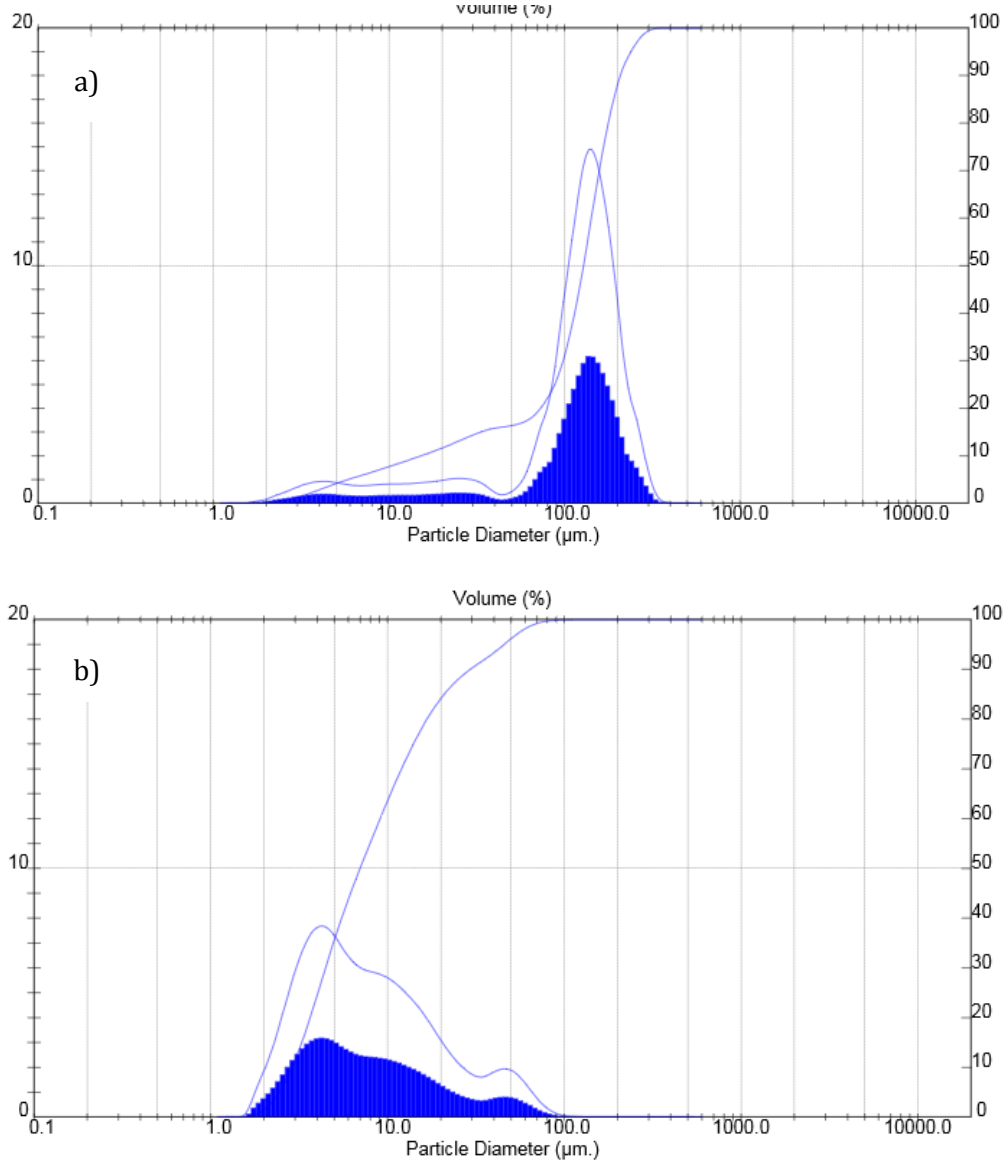


Figure 6.12. Particle size distribution of emulsion 20 a) cream layer and b) aqueous layer immediately after homogenisation. Emulsion 20 consisted of 20% oil, 0.5% yeast and no soap or broth.

There was a distinct difference in the particle sizes in the cream phase and the aqueous phase, as shown in (Figure 6.12) with the cream layer having larger droplets than the

aqueous layer, although both layers are polydisperse. The difference in size is due to the cream layer containing drop that have coalesced.

6.6 Conclusions

The FAME profile of the oil produced by *M. pulcherrima* varied substantially when the feedstock was varied from glucose to wheat straw, with the glucose-cultivated oil being more like palm olein and the wheat-straw cultivated oil being more like palm stearin. The surface tension of these oils was lower than that of palm oil, and greater than that of rapeseed oil.

The products of *M. pulcherrima* cultivation on wheat straw, i.e. *M. pulcherrima* cells (post-lipid extraction) and fermentation broth were found to provide some surfactant capability in that they lowered the interfacial tensions between rapeseed oil and water. When used in a real emulsion, this had only a small stabilising effect, more so for the yeast cell residue. The emulsions formed were mostly bimodal, and the lack of stability was probably due to the lack of stabilisation of the oil droplets, which allowed them to coalesce. The emulsions were most stable if large amounts of oil, soap and yeast residue were used, however they were not stable enough for a care product emulsion to be formed.

7 Conclusions

This work aimed to establish whether *M. pulcherrima* was a suitable organism for the production of lipids from waste resources. *M. pulcherrima* was found to grow on a range of sugars including pentoses. Importantly, it was grown under non-sterile conditions at pilot scale (500 L) with minimal temperature control, demonstrating that this yeast has the potential to reduce the cost of oil production from oleaginous yeast. The yeast could be made to behave oleaginously, producing a maximum of 47% lipid when grown on glycerol provided the temperature was reduced to 15 °C and nitrogen starvation could occur. The maximum lipid yield of 6.04 g L^{-1} was achieved on model lignocellulose hydrolysate using a fed-batch method.

Excitingly, it was found that *M. pulcherrima* could metabolise oligosaccharide mixtures, such as those produced from the partial depolymerisation of wheat straw. However, the high lipid content was not maintained when *M. pulcherrima* was cultured on these wheat straw hydrolysates, with a maximum lipid content of 26% and lipid yield of 1.12 g L^{-1} achieved. This was thought to be because of the lower quantity of sugar available and the presence of inhibitors, although *M. pulcherrima* was shown to have a good and wide-ranging tolerance to levels of individual inhibitors, generally higher than alternative yeasts in the literature.[110, 292]

Consolidated bioprocessing of wheat straw was demonstrated, utilising a pretreatment step of liquid hot water hydrolysis, after water was found to be the most effective acid in a screening of dilute acids. *M. pulcherrima* was able to compensate for the very low levels of

monosaccharide found in liquid hot water treated wheat straw by its ability to produce β-glucosidases. This work demonstrated *M. pulcherrima*'s ability to use these to degrade real lignocellulose hydrolysate for the first time, and suggested that this enzyme (or more likely, enzymes) were capable of hydrolysing oligoglucans longer than two glucose units. As a consequence, the cell dry mass of *M. pulcherrima* cells exceeded the concentration of mono and disaccharides in the hydrolysate. It was also found to produce the by-products 2-phenylethanol and arabitol in low quantities.

Attempts were made to increase the lipid content of *M. pulcherrima* by increasing the concentration of mono and oligo-saccharides in the hydrolysate using flow processes and microwave heating. The conventional heating yielded slightly more lipid than the more efficient microwave heating (26 % vs 24%), however the lipid yield was far higher (1.17 vs. 0.56 g L^{-1}). The culture conditions were also varied in an attempt to increase the lipid yield using design of experiments. The inoculum type and volume was found to be highly significant for both the cell dry mass and lipid content, and higher temperatures were found to be best for lipid yield, in contrast to the results for glucose and glycerol. It became apparent that although the models developed for the culture conditions to predict the cell dry mass improved were effective, the models lowered the lipid content and thus lipid yield when applied to a hydrolysate produced at a higher temperature. The carbon to nitrogen ratio appears to be the cause of this, as in the chemically defined glucose media, the level of C:N is at its highest point at the start then decreases as carbon is consumed, whereas for the hydrolysate the level of accessible carbon is initially lower and the yeast must first hydrolyse the oligosaccharides. This indicates that future analysis of culture conditions should be done on the specific hydrolysate. Another biomass source, seaweed, was shown to be utilisable by *M. pulcherrima* yielding 1.12 g L^{-1} lipid, despite containing sulfated sugars. Notwithstanding these attempts, the lipid yield was less than the target level of 2 g L^{-1}, as although the lipid content was in the oleaginous range (over 20%), the cell dry mass was less than the 13 g L^{-1} target, as was the lipid productivity (0.17 vs 0.7 g L^{-1} d^{-1}.)

The oil produced is ten times more viscous than palm oil, with a lower surface tension and energy density. The FAME profile varies depending on the carbon source, with glucose-cultivated oil being more like palm olein and the wheat-straw cultivated oil being more like palm stearin. Emulsions were formed using the yeast cell residue and fermentation broth, which were found to have surface active qualities, however these were insufficient

to stabilise the emulsions. If the oil were modified in-situ to a more effective surfactant, this, in combination with the broth and residue could allow for a simple route to a product without oil extraction.

Overall, this work has demonstrated that *M. pulcherrima* shows interesting potential for use in the industrial biotechnology, particularly in the more sustainable production of a palm oil-like substance from waste lignocellulose or glycerol. In order for the commercial production of a microbial oil to be successful, the estimated price must be reduced. The estimated cost of microbial oil in 2014 was estimated at US \$3.4 kg^{-1}, compared with a maximum of US \$ 1.2 kg^{-1} for palm oil. This estimate was based on a process using a glucose feedstock (US \$ 400 t^{-1}) and involving sterilisation. *M. pulcherrima*'s ability to produce cellulases, tolerate inhibitors, grow in non-sterile conditions with minimal temperature control and potentially produce multiple products from multiple carbon sources suggests a robustness and versatility that would be highly beneficial in reducing the capital and running costs of a putative production process. Fewer additional enzymes need be produced, which reduces the costs as well as the 40-60% of the global warming potential that enzyme production contributes.[59] This may allow utilisation of a theoretically more efficient consolidated bioprocessing approach from a waste lignocellulose feedstock (US \$0-80 t^{-1}).[63, 247] Only *C. curvatus* has been shown to grow and produce lipid in non-sterile conditions on a lignocellulose hydrolysate.[313] The ability to tolerate so many non-ideal conditions is unusual in a yeast species, with most demonstrating strengths in only a few areas[110, 142]

8 Future work

Although the lipid contents attained in this work were less than hoped, only three waste feedstocks were examined and varying yields of lipid were obtained with these. These data, and preliminary results on coffee waste, corn cob and food waste showed that different feedstocks could be more suitable. Future work would involve a more thorough screen of other feedstocks, and also of fermentation time. A successful strategy for improving the lipid yield has been to examine other strains, and other yeast species in the same family.[123, 125, 127, 128] *Metschnikowia sp.* are extremely common worldwide and such different habitats give rise to multiple strains. For example, 260 *Metschnikowia* strains were isolated from grapes and grape must in one study in Sardinia.[316] This genetic diversity may be harnessed to screen a robust strain that also produces higher quantities of lipid, or other useful products.

If consumer attitudes and regulatory regimes change, metabolic engineering of *M. pulcherrima* may be a viable way to increase the yield of lipid, such as the modification of *Y. lipolytica* that led to a lipid yield of 18 g L^{-1} from acid pretreated corn stover.[143] Genetic manipulation could also potentially be used to create other valuable products.[392] Preliminary results suggest that *M. pulcherrima* is amenable to genetic manipulation.

The optimal bioreactor design and method for *M. pulcherrima* cultivation has not been established. Oxygen supply is likely to be an important factor as this is required for lipid synthesis, however air compressors are one of the most costly pieces of equipment (ca. US $1 m per unit), along with fermenters (ca. US $3 m per unit).[191] Preventing overheating

would also be important in a large scale fermentation. Airlift fermenters provide solutions to these problems, as they require a lower pressure of air (and so a less powerful and cheaper compressor), and provide thorough agitation as well as cooling.[393, 394]

Once the lipid yield is at a more acceptable level, in-situ conversion of lipid to fatty acid esters of polyethylene glycol should be investigated. There may be difficulties in ensuring the polyethylene glycol can reach the oils, perhaps necessitating high temperatures to rupture the cells. The protein components of the yeast cell residue could be made more effective by optimising the pH and salt content, as proteins are most effective at stabilising emulsions outside their isoelectric point. Other ways of thickening care product emulsions involve using partially oxidised cellulose. As more hemicellulose was hydrolysed than cellulose, the residue from the hydrolysis process is rich in cellulose, and could perhaps be used to generate this component. Other surfactants than soaps could also be made from the triglycerides, such as polyethylene glycol polymers, which could be more effective.

M. pulcherrima has potential to be combined with other processes. It could be used in wastewater treatment, removing the nutrients and oils from the water to create new surfactants concentrated within the cells.[298] *M. pulcherrima* could be integrated into a biorefinery, in which the yeast generates products from the aqueous phase of microwave pyrolysed lignocellulose.[65] The classic example of improving the yields from biodiesel production by converting glycerol to triglyceride and then transesterifying this to the product is clearly a possibility.[106] Overall, *M. pulcherrima* has the potential to contribute to a more sustainable source of many products, from biofuels and care products to fine chemicals and food.

9 References

1. OECD/FAO, *OECD-FAO Agricultural Outlook 2014*, 1st edn., OECD Publishing.
2. Roundtable on Sustainable Palm Oil, *About sustainable palm oil*, http://www.rspo.org/consumers/about-sustainable-palm-oil, Accessed 14/05/2015, 2015.
3. Y. Basiron, *Palm oil production through sustainable plantations*, Eur. J. Lipid Sci. Technol., 2007, **109**, 289-295.
4. V. Tauli-Corpuz and P. Tamang, *Primary Oil Palm and Other Commercial Tree Plantations, Monocropping: Impacts on Indigenous Peoples' Land Tenure and Resource Management Systems and Livelihoods.*, ed. People's forum on Indiginous Issues, United Nations, New York, 2007.
5. M. Broich, M. Hansen, F. Stolle, P. Potapov, B. A. Margono and B. Adusei, *Remotely sensed forest cover loss shows high spatial and temporal variation across Sumatera and Kalimantan, Indonesia 2000-2008*, Environ. Res. Lett., 2011, **6**.
6. B. A. Margono, S. Turubanova, I. Zhuravleva, P. Potapov, A. Tyukavina, A. Baccini, S. Goetz and M. C. Hansen, *Mapping and monitoring deforestation and forest degradation in Sumatra (Indonesia) using Landsat time series data sets from 1990 to 2010*, Environ. Res. Lett., 2012, **7**.
7. D. A. Ehlers Smith, *The effects of land-use policies on the conservation of Borneo's endemic Presbytis monkeys*, Biodivers Conserv, 2014, **23**, 891-908.
8. J. Miettinen, J. Wang, A. Hooijer and S. Liew, *Peatland conversion and degradation processes in insular southeast asia: A case study in Jambi, Indonesia*, Land Degrad. Dev., 2013, **24**, 334-341.
9. N. S. Sodhi, L. P. Koh, B. W. Brook and P. K. L. Ng, *Southeast Asian biodiversity: an impending disaster*, Trends Ecol Evol, 2004, **19**, 654-660.
10. K. Hergoualc'h and L. V. Verchot, *Greenhouse gas emission factors for land use and land-use change in Southeast Asian peatlands*, Mitigation and Adaptation Strategies for Global Change, 2014, **19**, 789-807.

11. J. Fargione, J. Hill, D. Tilman, S. Polasky and P. Hawthorne, *Land clearing and the biofuel carbon debt*, Science, 2008, **319**, 1235-1238.

12. G. Bala, K. Caldeira, M. Wickett, T. J. Phillips, D. B. Lobell, C. Delire and A. Mirin, *Combined climate and carbon-cycle effects of large-scale deforestation*, Proceedings of the National Academy of Sciences, 2007, **104**, 6550-6555.

13. I. C. W. T. A. R. K. P. a. A. Reisinger, *Primary Climate Change 2007: Synthesis Report. Contribution of Working Groups I, II and III to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*, Intergovernemntal Panel on Climate Change, Geneva, Switzerland, 2007, p. 104.

14. M. C. Hansen, P. V. Potapov, R. Moore, M. Hancher, S. A. Turubanova, A. Tyukavina, D. Thau, S. V. Stehman, S. J. Goetz, T. R. Loveland, A. Kommareddy, A. Egorov, L. Chini, C. O. Justice and J. R. G. Townshend, *High-Resolution Global Maps of 21st-Century Forest Cover Change*, Science, 2013, **342**, 850-853.

15. F. Ramdani and M. Hino, *Land use changes and GHG emissions from tropical forest conversion by oil palm plantations in Riau Province, Indonesia*, PLoS One, 2013, **8**, e70323.

16. Unilever, *Sustainable palm oil progress report 2014*, Rotterdam/London, 2014.

17. X. Zhang, L. Li, H. Xie, Z. Liang, J. Su, G. Liu and B. Li, *Comparative Analysis of Thermal Behavior, Isothermal Crystallization Kinetics and Polymorphism of Palm Oil Fractions*, Molecules, 2013, **18**, 1036-1052.

18. J. Kohlndorfer, *An Improved Artificial Fuel, and Process for Producing the same, Great Britain Pat.*, GB189705669, 1896.

19. E. Commission, *Primary Amending Decision 96/335/EC establishing an inventory and a common nomenclature of ingredients employed in cosmetic products*, ed. THE COMMISSION OF THE EUROPEAN COMMUNITIES, Official Journal of the European Union, Brussels, 2006.

20. M. Randle, *SKIN APPLICATION*, WO2007093830, 2007.

21. D. J. Shaw, *Introduction to colloid and surface chemistry*, 1st edn., Oxford : Butterworth-Heinemann, Oxford, 1992.

22. P. W. Atkins, *Elements of physical chemistry*, 1st edn., Oxford : Oxford University Press, Oxford, 2005.

23. J. M. Ageitos, J. A. Vallejo, P. Veiga-Crespo and T. G. Villa, *Oily yeasts as oleaginous cell factories*, Appl. Microbiol. Biotechnol., 2011, **90**, 1219-1227.

24. B. S. Saharan, R. K. Sahu and D. Sharma, *A review on biosurfactants: Fermentation, current developments and perspectives*, Genetic Engineering and Biotechnology Journal, 2012, 1-14.

25. R. Marchant and I. M. Banat, *Microbial biosurfactants: Challenges and opportunities for future exploitation*, Trends Biotechnol., 2012, **30**, 558-565.

26. R. Marchant and I. M. Banat, *Biosurfactants: A sustainable replacement for chemical surfactants?*, Biotechnol. Lett., 2012, **34**, 1597-1605.

27. D. Develter and S. Fleurackers, *Method For The Production Of Medium-Chain Sophorolipid, US8530206* 2008.

28. Z. Aksu and A. T. Eren, *Production of carotenoids by the isolated yeast of Rhodotorula glutinis*, Biochem Eng J, 2007, **35**, 107-113.

29. C. Saenge, B. Cheirsilp, T. T. Suksaroge and T. Bourtoom, *Potential use of oleaginous red yeast Rhodotorula glutinis for the bioconversion of crude glycerol from biodiesel plant to lipids and carotenoids*, Process Biochem, 2011, **46**, 210-218.

30. A. Corrêa do Lago, A. Bonomi, O. Cavalett, M. Pereira da Cunha and M. A. Pinheiro Lima, *Sugarcane as a carbon source: The Brazilian case*, Biomass Bioenergy, 2012, **46**, 5-12.

31. C. J. Chuck, F. Santomauro, L. A. Sargeant, F. Whiffin, T. Chantasuban, N. R. Abdul Ghaffar, J. L. Wagner and R. J. Scott, *Liquid transport fuels from microbial yeasts - current and future perspectives*, Biofuels, 2014, **5**, 293-311.

32. M. J. Groom, E. M. Gray and P. A. Townsend, *Biofuels and biodiversity: Principles for creating better policies for biofuel production*, Conserv Biol, 2008, **22**, 602-609.

33. L. P. Koh and J. Ghazoul, *Biofuels, biodiversity, and people: Understanding the conflicts and finding opportunities*, Biol Conserv, 2008, **141**, 2450-2460.

34. P. Kumar, D. M. Barrett, M. J. Delwiche and P. Stroeve, *Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production*, Ind. Eng. Chem. Res., 2009, **48**, 3713-3729.

35. A. K. Chandel, S. S. da Silva and O. V. Singh, *Detoxification of Lignocellulose Hydrolysates: Biochemical and Metabolic Engineering Toward White Biotechnology*, Bioenergy Research, 2013, **6**, 388-401.

36. W. M. J. Achten, J. Almeida, V. Fobelets, E. Bolle, E. Mathijs, V. P. Singh, D. N. Tewari, L. V. Verchot and B. Muys, *Life cycle assessment of Jatropha biodiesel as transportation fuel in rural India*, Appl. Energy, 2010, **87**, 3652-3660.

37. N. Contran, L. Chessa, M. Lubino, D. Bellavite, P. P. Roggero and G. Enne, *State-of-the-art of the Jatropha curcas productive chain: From sowing to biodiesel and by-products*, Ind. Crops Prod., 2013, **42**, 202-215.

38. Y. Chisti, *Biodiesel from microalgae*, Biotechnol Adv, 2007, **25**, 294-306.

39. M. A. Borowitzka, *Commercial production of microalgae: ponds, tanks, tubes and fermenters*, J. Biotechnol., 1999, **70**, 313-321.

40. L. Brennan and P. Owende, *Biofuels from microalgae-A review of technologies for production, processing, and extractions of biofuels and co-products*, Renewable Sustainable Energy Rev., 2010, **14**, 557-577.

41. Y. Chisti, *Biodiesel from microalgae beats bioethanol*, Trends Biotechnol., 2008, **26**, 126-131.

42. Q. C. Doan, N. R. Moheimani, A. J. Mastrangelo and D. M. Lewis, *Microalgal biomass for bioethanol fermentation: Implications for hypersaline systems with an industrial focus*, Biomass Bioenergy, 2012, **46**, 79-88.

43. R. Halim, M. K. Danquah and P. A. Webley, *Extraction of oil from microalgae for biodiesel production: A review*, Biotechnol Adv, 2012, **30**, 709-732.

44. M. K. Lam and K. T. Lee, *Microalgae biofuels: A critical review of issues, problems and the way forward*, Biotechnol Adv, 2012, **30**, 673-690.

45. A. K. Lee, D. M. Lewis and P. J. Ashman, *Disruption of microalgal cells for the extraction of lipids for biofuels: Processes and specific energy requirements*, Biomass Bioenergy, 2012, **46**, 89-101.

46. T. M. Mata, A. A. Martins and N. S. Caetano, *Microalgae for biodiesel production and other applications: A review*, Renewable Sustainable Energy Rev., 2010, **14**, 217-232.

47. L. Rodolfi, G. C. Zittelli, N. Bassi, G. Padovani, N. Biondi, G. Bonini and M. R. Tredici, *Microalgae for oil: Strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor*, Biotechnol. Bioeng., 2009, **102**, 100-112.

48. P. Spolaore, C. Joannis-Cassan, E. Duran and A. Isambert, *Commercial applications of microalgae*, J. Biosci. Bioeng., 2006, **101**, 87-96.

49. A. L. Mascarelli, *Algae: fuel of the future?*, Environ Sci Technol, 2009, **43**, 7160-7161.

50. C. Ratledge and Z. Cohen, *Microbial and algal oils: Do they have a future for biodiesel or as commodity oils?*, Lipid Technology, 2008, **20**, 155-160.

51. Y. Gao, C. Gregor, Y. Liang, D. Tang and C. Tweed, *Algae biodiesel - a feasibility report*, Chem. Cent. J., 2012, **6**, S1.

52. S. D. Ríos, J. Castañeda, C. Torras, X. Farriol and J. Salvadó, *Lipid extraction methods from microalgal biomass harvested by two different paths: Screening studies toward biodiesel production*, Bioresour. Technol., 2013, **133**, 378-388.

53. *Exxon takes algae biofuels programme back to the drawing board after $100 million spend*, Int. Sugar J., 2013, **115**, 462-462.

54. D. Pirozzi, A. Ausiello, R. Strazza, M. Trofa, G. Zuccaro and G. Toscano, *Primary Exploitation of agricultural biomasses to produce iigeneration biodiesel* in Chemical Engineering Transactions, 2013, vol. 32, pp. 175-180.

55. P. Roy, K. Tokuyasu, T. Orikasa, N. Nakamura and T. Shiina, *A Review of Life Cycle Assessment (LCA) of Bioethanol from Lignocellulosic Biomass*, Jarq-Japan Agricultural Research Quarterly, 2012, **46**, 41-57.

56. N. Sarkar, S. K. Ghosh, S. Bannerjee and K. Aikat, *Bioethanol production from agricultural wastes: An overview*, Renewable Energy, 2012, **37**, 19-27.

57. P. Thornley, *Clean Energy from Rice Straw*, http://www.supergen-bioenergy.net/research-projects/rice-straw-project/, Accessed 09/07/2015, 2015.

58. F. Talebnia, D. Karakashev and I. Angelidaki, *Production of bioethanol from wheat straw: An overview on pretreatment, hydrolysis and fermentation*, Bioresour. Technol., 2010, **101**, 4744-4753.

59. L. Wang, J. Littlewood and R. J. Murphy, *Environmental sustainability of bioethanol production from wheat straw in the UK*, Renewable Sustainable Energy Rev., 2013, **28**, 715-725.

60. US Department of Energy, *Primary U.S. billion ton update: biomass for a bioenergy and bioproducts industry*, ed. Energy, US Department of Energy, Washington, 2011.

61. C. M. Pete Harrison, Stephanie Searle, Anil Baral, David Turley, Lucy Hopwood, *Wasted: Europe's untapped resource*, The International Council on Clean Transportation, 2014.

62. C. O. Tuck, E. Pérez, I. T. Horváth, R. A. Sheldon and M. Poliakoff, *Valorization of biomass: Deriving more value from waste*, Science, 2012, **337**, 695-699.

63. World Bank, *Primary Global Economic Monitor (GEM) Commodities*, ed. W. Bank, World Databank, 2015.

64. R. M. Rodriguez-Jasso, S. I. Mussatto, L. Pastrana, C. N. Aguilar and J. A. Teixeira, *Microwave-assisted extraction of sulfated polysaccharides (fucoidan) from brown seaweed*, Carbohydr. Polym., 2011, **86**, 1137-1144.

65. V. L. Budarin, P. S. Shuttleworth, J. R. Dodson, A. J. Hunt, B. Lanigan, R. Marriott, K. J. Milkowski, A. J. Wilson, S. W. Breeden, J. Fan, E. H. K. Sin and J. H. Clark, *Use of green chemical technologies in an integrated biorefinery*, Energy Environ. Sci., 2011, **4**, 471-479.

66. A. Pometto, K. Shetty, G. Paliyath and R. E. Levin, *Food Biotechnology, Second Edition*, 1st edn., Taylor & Francis, 2005.

67. D. J. A. Crommelin, R. D. Sindelar, B. Meibohm, *Pharmaceutical Biotechnology: Fundamentals and Applications*, 3rd edn., Springer, New York, NY, 2013.

68. J. C. Escobar, E. S. Lora, O. J. Venturini, E. E. Yanez, E. F. Castillo and O. Almazan, *Biofuels: Environment, technology and food security*, Renewable Sustainable Energy Rev., 2009, **13**, 1275-1287.

69. Q. Li, W. Du and D. Liu, *Perspectives of microbial oils for biodiesel production*, Appl. Microbiol. Biotechnol., 2008, **80**, 749-756.

70. A. Tanimura, M. Takashima, T. Sugita, R. Endoh, M. Kikukawa, S. Yamaguchi, E. Sakuradani, J. Ogawa and J. Shima, *Selection of oleaginous yeasts with high lipid productivity for practical biodiesel production*, Bioresour. Technol., 2014, **153**, 230-235.

71. T. Roukas, in *Food Biotechnology, Second Edition*, eds. A. Pometto, K. Shetty, G. Paliyath and R. E. Levin, Taylor & Francis, 2nd edn., 2005, pp. 433-472.

72. J. Sheehan, T. Dunahay, J. Benemann and P. Roessler, *A Look Back at the U.S. Department of Energy's Aquatic Species Program: Biodiesel from Algae* National Renewable Energy Laboratory, 1998.

73. C. Huang, X.-f. Chen, L. Xiong, X.-d. Chen, L.-l. Ma and Y. Chen, *Single cell oil production from low-cost substrates: The possibility and potential of its industrialization*, Biotechnol Adv, 2013, **31**, 129-139.

74. J. P. Delgenes, R. Moletta and J. M. Navarro, *Effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by Saccharomyces cerevisiae, Zymomonas mobilis, Pichia stipitis, and Candida shehatae*, Enzyme Microb. Technol., 1996, **19**, 220-225.

75. T. Sugita and M. Takashima, *Yeasts, A Taxonomic Study*, Japanese Journal of Medical Mycology, 2011, **52**, 107-115.

76. M.-A. Lachance, in *Biodiversity and Ecophysiology of Yeasts*, eds. G. Péter and C. Rosa, Springer Berlin Heidelberg, Editon edn., 2006, pp. 1-9.

77. G. b. a. R. Péter, Carlos, *Biodiversity and Ecophysiology of Yeasts*, 1st edn., Berlin, Heidelberg : Springer-Verlag Berlin Heidelberg, Berlin, Heidelberg, 2006.

78. I. R. Sitepu, L. A. Garay, R. Sestric, D. Levin, D. E. Block, J. B. German and K. L. Boundy-Mills, *Oleaginous yeasts for biodiesel: Current and future trends in biology and production*, Biotechnol Adv, 2014, **32**, 1336-1360.

79. F. Donot, A. Fontana, J. C. Baccou, C. Strub and S. Schorr-Galindo, *Single cell oils (SCOs) from oleaginous yeasts and moulds: Production and genetics*, Biomass Bioenergy, 2014, **68**, 135-150.

80. C. Ratledge, *Single cell oils — have they a biotechnological future?*, Trends Biotechnol., 1993, **11**, 278-284.

81. S. Papanikolaou and G. Aggelis, *Lipids of oleaginous yeasts. Part I: Biochemistry of single cell oil production*, Eur. J. Lipid Sci. Technol., 2011, **113**, 1031-1051.

82. P. Meeuwse, J. P. M. Sanders, J. Tramper and A. Rinzema, *Lipids from yeasts and fungi: Tomorrow's source of biodiesel?*, Biofuels, Bioproducts and Biorefining, 2013, **7**, 512-524.

83. S. Papanikolaou and G. Aggelis, *Lipids of oleaginous yeasts. Part II: Technology and potential applications*, Eur. J. Lipid Sci. Technol., 2011, **113**, 1052-1073.

84. I. R. Sitepu, R. Sestric, L. Ignatia, D. Levin, J. B. German, L. A. Gillies, L. A. G. Almada and K. L. Boundy-Mills, *Manipulation of culture conditions alters lipid content and fatty acid profiles of a wide variety of known and new oleaginous yeast species*, Bioresour. Technol., 2013, **144**, 360-369.

85. K. Kavanagh and J. Kelly, in *Hugo and Russell's pharmaceutical microbiology*, eds. W. B. Hugo, S. P. Denyer, N. Hodges, S. Gorman and B. Gilmore, Chichester, West Sussex, UK, 2011.

86. J. I. Pitt and M. W. Miller, *Sporulation in Candida pulcherrima, Candida Reukaufii and Chlamydozyma Species: Their Relationships with Metschnikowia*, Mycologia, 1968, **60**, 663-685.

87. E. M. T. El-Mansi, F. B. Ward and P. C. Arun, in *Fermentation Microbiology and Biotechnology, Third Edition*, ed. A. Allman, CRC Press, Editon edn., 2011, pp. 9-36.

88. C. Ratledge, in *Industrial applications of single cell oils*, ed. D. J. K. a. C. Ratledge, American Oil Chemists Society, Boulder, Urbana, IL, Editon edn., 1993.

89. C. Ratledge, *Yeasts For Lipid Production*, Biochem. Soc. Trans., 1988, **16**, 1088-1091.

90. Y. Li, Z. Zhao and F. Bai, *High-density cultivation of oleaginous yeast Rhodosporidium toruloides Y4 in fed-batch culture*, Enzyme Microb. Technol., 2007, **41**, 312-317.

91. C. Angerbauer, M. Siebenhofer, M. Mittelbach and G. M. Guebitz, *Conversion of sewage sludge into lipids by Lipomyces starkeyi for biodiesel production*, Bioresour. Technol., 2008, **99**, 3051-3056.

92. S. Bellou, A. Moustogianni, A. Makri and G. Aggelis, *Lipids containing polyunsaturated fatty acids synthesized by zygomycetes grown on glycerol*, Appl Biochem Biotechnol, 2012, **166**, 146-158.

93. H. E. Bialy, O. M. Gomaa and K. S. Azab, *Conversion of oil waste to valuable fatty acids using Oleaginous yeast*, World J. Microbiol. Biotechnol., 2011, **27**, 2791-2798.

94. C. A. Boulton and C. Ratledge, *Cryptococcus terricolus, an oleaginous yeast re-appraised*, Appl. Microbiol. Biotechnol., 1984, **20**, 72-76.

95. A. Chatzifragkou, A. Makri, A. Belka, S. Bellou, M. Mavrou, M. Mastoridou, P. Mystrioti, G. Onjaro, G. Aggelis and S. Papanikolaou, *Biotechnological conversions of biodiesel derived waste glycerol by yeast and fungal species*, Energy, 2011, **36**, 1097-1108.

96. S. H. Duarte, C. C. P. de Andrade, G. Ghiselli and F. Maugeri, *Exploration of Brazilian biodiversity and selection of a new oleaginous yeast strain cultivated in raw glycerol*, Bioresour. Technol., 2013, **138**, 377-381.

97. V. Johnson, M. Singh, V. S. Saini, V. R. Sista and N. K. Yadav, *Effect of pH on lipid accumulation by an oleaginous yeast:Rhodotorula glutinis IIP-30*, World J Microbiol Biotechnol, 1992, **8**, 382-384.

98. Y. Jigami, O. Suzuki and S. Nakasato, *Comparison of lipid composition of Candida guilliermondii grown on glucose, ethanol and methanol as the sole carbon source*, Lipids, 1979, **14**, 937-942.

99. P. Rathi, R. K. Saxena and R. Gupta, *A novel alkaline lipase from Burkholderia cepacia for detergent formulation*, Process Biochem, 2001, **37**, 187-192.

100. A. Ykema, E. C. Verbree, M. M. Kater and H. Smit, *Optimization of lipid production in the oleaginous yeast Apiotrichum curvatum in wheypermeate*, Appl. Microbiol. Biotechnol., 1988, **29**, 211-218.

101. G. Venkata Subhash and S. Venkata Mohan, *Biodiesel production from isolated oleaginous fungi Aspergillus sp. using corncob waste liquor as a substrate*, Bioresour. Technol., 2011, **102**, 9286-9290.

102. M. Zhu, L. J. Yu, W. Li, P. P. Zhou and C. Y. Li, *Optimization of arachidonic acid production by fed-batch culture of Mortierella alpina based on dynamic analysis*, Enzyme Microb. Technol., 2006, **38**, 735-740.

103. A. André, P. Diamantopoulou, A. Philippoussis, D. Sarris, M. Komaitis and S. Papanikolaou, *Biotechnological conversions of bio-diesel derived waste glycerol into added-value compounds by higher fungi: production of biomass, single cell oil and oxalic acid*, Ind. Crops Prod., 2010, **31**, 407-416.

104. L. Hui, C. Wan, D. Hai-Tao, C. Xue-Jiao, Z. Qi-Fa and Z. Yu-Hua, *Direct microbial conversion of wheat straw into lipid by a cellulolytic fungus of Aspergillus oryzae A-4 in solid-state fermentation*, Bioresour. Technol., 2010, **101**, 7556-7562.

105. S. Papanikolaou and G. Aggelis, *Yarrowia lipolytica: A model microorganism used for the production of tailor-made lipids*, Eur. J. Lipid Sci. Technol., 2010, **112**, 639-654.

106. S. Fakas, S. Papanikolaou, A. Batsos, M. Galiotou-Panayotou, A. Mallouchos and G. Aggelis, *Evaluating renewable carbon sources as substrates for single cell oil production by Cunninghamella echinulata and Mortierella isabellina*, Biomass Bioenergy, 2009, **33**, 573-580.

107. D. Mitra, M. L. Rasmussen, P. Chand, V. R. Chintareddy, L. Yao, D. Grewell, J. G. Verkade, T. Wang and J. Hans van Leeuwen, *Value-added oil and animal feed production from corn-ethanol stillage using the oleaginous fungus Mucor circinelloides*, Bioresour. Technol., 2012, **107**, 368-375.

108. P. Xiaowei and C. Hongzhang, *Hemicellulose sugar recovery from steam-exploded wheat straw for microbial oil production*, Process Biochem, 2012, **47**, 209-215.

109. L. C. Duarte, F. Carvalheiro, S. Lopes, I. Neves and F. M. Girio, *Yeast biomass production in brewery's spent grains hemicellulosic hydrolyzate*, Appl. Biochem. Biotechnol., 2008, **148**, 119-129.

110. I. Sitepu, T. Selby, T. Lin, S. Zhu and K. Boundy-Mills, *Carbon source utilization and inhibitor tolerance of 45 oleaginous yeast species*, J. Ind. Microbiol. Biotechnol., 2014, **41**, 1061-1070.

111. A. Amaretti, S. Raimondi, A. Leonardi and M. Rossi, *Primary Candida freyschussii: An oleaginous yeast producing lipids from glycerol* in Chemical Engineering Transactions, 2012, vol. 27, pp. 139-144.

112. C. O. Gill, M. J. Hall and C. Ratledge, *Lipid accumulation in an oleaginous yeast (Candida 107) growing on glucose in single stage continuous culture*, Appl. Environ. Microbiol., 1977, **33**, 231-239.

113. G. Munch, R. Sestric, R. Sparling, D. B. Levin and N. Cicek, *Lipid production in the under-characterized oleaginous yeasts, Rhodosporidium babjevae and Rhodosporidium diobovatum, from biodiesel-derived waste glycerol*, Bioresour. Technol., 2015, **185**, 49-55.

114. S. Franklin, J. Piechocki, L. M. Norris, S. M. Decker, J. Wee and D. Zdanis, *Oleaginous yeast food compositions*, WO2011130576, 2011.

115. L. Hansson and M. Dostalek, *Influence of cultivation conditions on lipid production by Cryptococcus albidus*, Appl. Microbiol. Biotechnol., 1986, **24**, 12-18.

116. F. Yang, S. Zhang, W. Tang and Z. K. Zhao, *Identification of the orotidine-5′ - monophosphate decarboxylase gene of the oleaginous yeast Rhodosporidium toruloides*, Yeast, 2008, **25**, 623-630.

117. Q. Wang, F. J. Guo, Y. J. Rong and Z. M. Chi, *Lipid production from hydrolysate of cassava starch by Rhodosporidium toruloides 21167 for biodiesel making*, Renewable Energy, 2012, **46**, 164-168.

118. Z. Chi, Y. Zheng, J. Ma and S. Chen, *Oleaginous yeast Cryptococcus curvatus culture with dark fermentation hydrogen production effluent as feedstock for microbial lipid production*, Int. J. Hydrogen Energy, 2011, **36**, 9542-9550.

119. H. Uemura, *Synthesis and production of unsaturated and polyunsaturated fatty acids in yeast: Current state and perspectives*, Appl. Microbiol. Biotechnol., 2012, **95**, 1-12.

120. M. Thiru, S. Sankh and V. Rangaswamy, *Process for biodiesel production from Cryptococcus curvatus*, Bioresour. Technol., 2011, **102**, 10436-10440.

121. E. Uçkun Kiran, A. Salakkam, A. P. Trzcinski, U. Bakir and C. Webb, *Enhancing the value of nitrogen from rapeseed meal for microbial oil production*, Enzyme Microb. Technol., 2012, **50**, 337-342.

122. L. Heredia and C. Ratledge, *Simultaneous utilization of glucose and xylose by Candida curvata D in continuous culture*, Biotechnol. Lett., 1988, **10**, 25-30.

123. A. Amaretti, S. Raimondi, M. Sala, L. Roncaglia, M. De Lucia, A. Leonardi and M. Rossi, *Single cell oils of the cold-adapted oleaginous yeast Rhodotorula glacialis DBVPG 4785*, Microb Cell Fact, 2010, **9**.

124. M. Hassan, P. J. Blanc, L. M. Granger, A. Pareilleux and G. Goma, *Lipid production by an unsaturated fatty acid auxotroph of the oleaginous yeast Apiotrichum curvatum grown in single-stage continuous culture*, Appl. Microbiol. Biotechnol., 1993, **40**, 483-488.

125. I. Sitepu, M. Jin, J. E. Fernandez, L. da Costa Sousa, V. Balan and K. Boundy-Mills, *Identification of oleaginous yeast strains able to accumulate high intracellular lipids when cultivated in alkaline pretreated corn stover*, Appl. Microbiol. Biotechnol., 2014, **98**, 7645-7657.

126. Z. Jacob, *Enrichment of wheat bran by Rhodotorula gracilis through solid-state fermentation*, Folia Microbiol, 1991, **36**, 86-91.

127. S. Galafassi, D. Cucchetti, F. Pizza, G. Franzosi, D. Bianchi and C. Compagno, *Lipid production for second generation biodiesel by the oleaginous yeast Rhodotorula graminis*, Bioresour. Technol., 2012, **111**, 398-403.

128. L. A. M. Sargeant, Matthew; Saad-Allah, Khalil M.; Hussein, Ali H.; Whiffin, Fraeya; Santomauro, Fabio; Chuck, Christopher J., *Production of lipid from depolymerised lignocellulose using the biocontrol yeast, Rhodotorula minuta: The fatty acid profile remains stable irrespective of environmental conditions*, Eur. J. Lipid Sci. Technol., 2015, **117**, In press.

129. I. R. Sitepu, L. Ignatia, A. K. Franz, D. M. Wong, S. A. Faulina, M. Tsui, A. Kanti and K. Boundy-Mills, *An improved high-throughput Nile red fluorescence assay for estimating intracellular lipids in a variety of yeast species*, J Microbiol Methods, 2012, **91**, 321-328.

130. C. H. Zhao, T. Zhang, M. Li and Z. M. Chi, *Single cell oil production from hydrolysates of inulin and extract of tubers of Jerusalem artichoke by Rhodotorula mucilaginosa TJY15a*, Process Biochem, 2010, **45**, 1121-1126.

131. Y. Cui, C. Fraser, G. Gardner, C. J. Huang, M. Reith and A. J. Windust, *Isolation and optimisation of the oleaginous yeast Sporobolomyces roseus for biosynthesis of 13C isotopically labelled 18-carbon unsaturated fatty acids and trans 18:1 and 18:2 derivatives through synthesis*, J. Ind. Microbiol. Biotechnol., 2012, **39**, 153-161.

132. C. Huang, X. F. Chen, L. Xiong, X. Y. Yang, X. D. Chen, L. L. Ma and Y. Chen, *Microbial oil production from corncob acid hydrolysate by oleaginous yeast Trichosporon coremiiforme*, Biomass Bioenergy, 2013, **49**, 273-278.

133. Q. Gao, Z. Cui, J. Zhang and J. Bao, *Lipid fermentation of corncob residues hydrolysate by oleaginous yeast Trichosporon cutaneum*, Bioresour. Technol., 2014, **152**, 552-556.

134. M. Rossi, P. Buzzini, L. Cordisco, A. Amaretti, M. Sala, S. Raimondi, C. Ponzoni, U. M. Pagnoni and D. Matteuzzi, *Growth, lipid accumulation, and fatty acid composition in obligate psychrophilic, facultative psychrophilic, and mesophilic yeasts*, FEMS Microbiol. Ecol., 2009, **69**, 363-372.

135. C. Huang, X. F. Chen, L. Xiong, X. D. Chen and L. L. Ma, *Oil production by the yeast Trichosporon dermatis cultured in enzymatic hydrolysates of corncobs*, Bioresour. Technol., 2012, **110**, 711-714.

136. E. Oguri, K. Masaki, T. Naganuma and H. Iefuji, *Phylogenetic and biochemical characterization of the oil-producing yeast Lipomyces starkeyi*, Antonie van Leeuwenhoek, , 2012, **101**, 359-368.

137. X. Yu, Y. Zheng, K. M. Dorgan and S. Chen, *Oil production by oleaginous yeasts using the hydrolysate from pretreatment of wheat straw with dilute sulfuric acid*, Bioresour. Technol., 2011, **102**, 6134-6140.

138. Y. A. Tsigie, C.-Y. Wang, T. Chi-Thanh and Y.-H. Ju, *Lipid production from Yarrowia lipolytica Po1g grown in sugarcane bagasse hydrolysate*, Bioresour. Technol., 2011, **102**, 9216-9222.

139. S. Papanikolaou and G. Aggelis, *Lipid production by Yarrowia lipolytica growing on industrial glycerol in a single-stage continuous culture*, Bioresour. Technol., 2002, **82**, 43-49.

140. S. Papanikolaou, I. Chevalot, M. Komaitis, I. Marc and G. Aggelis, *Single cell oil production by Yarrowia lipolytica growing on an industrial derivative of animal fat in batch cultures*, Appl. Microbiol. Biotechnol., 2002, **58**, 308-312.

141. C. T. Evans and C. Ratledge, *A comparison of the oleaginous yeast, Candida curvata, grown on different carbon sources in continuous and batch culture*, Lipids, 1983, **18**, 623-629.

142. L. Y. Zhu, M. H. Zong and H. Wu, *Efficient lipid production with Trichosporon fermentans and its use for biodiesel preparation*, Bioresour. Technol., 2008, **99**, 7881-7885.

143. L. X. Pan, D. F. Yang, L. Shao, W. Li, G. G. Chen and Z. Q. Liang, *Isolation of the oleaginous yeasts from the soil and studies of their lipid-producing capacities*, Food Technol Biotechnol, 2009, **47**, 215-220.

144. K. Qiao, S. H. Imam Abidi, H. Liu, H. Zhang, S. Chakraborty, N. Watson, P. Kumaran Ajikumar and G. Stephanopoulos, *Engineering lipid overproduction in the oleaginous yeast Yarrowia lipolytica*, Metab. Eng., 2015, **29**, 56-65.

145. D. Xie, E. N. Jackson and Q. Zhu, *Sustainable source of omega-3 eicosapentaenoic acid from metabolically engineered Yarrowia lipolytica: from fundamental research to commercial production*, Appl. Microbiol. Biotechnol., 2015, **99**, 1599-1610.

146. W. Wang, H. Wei, M. Alahuhta, X. Chen, D. Hyman, D. K. Johnson, M. Zhang and M. E. Himmel, *Heterologous Expression of Xylanase Enzymes in Lipogenic Yeast <italic>Yarrowia lipolytica</italic>*, PLoS One, 2014, **9**, e111443.

147. B. Cheirsilp and Y. Louhasakul, *Industrial wastes as a promising renewable source for production of microbial lipid and direct transesterification of the lipid into biodiesel*, Bioresour. Technol., 2013, **142**, 329-337.

148. M. Tai and G. Stephanopoulos, *Engineering the push and pull of lipid biosynthesis in oleaginous yeast Yarrowia lipolytica for biofuel production*, Metab. Eng., 2013, **15**, 1-9.

149. A. Makri, S. Fakas and G. Aggelis, *Metabolic activities of biotechnological interest in Yarrowia lipolytica grown on glycerol in repeated batch cultures*, Bioresour. Technol., 2010, **101**, 2351-2358.

150. A. Beopoulos, T. Chardot and J.-M. Nicaud, *Yarrowia lipolytica: A model and a tool to understand the mechanisms implicated in lipid accumulation*, Biochimie, 2009, **91**, 692-696.

151. A. Beopoulos, J. Cescut, R. Haddouche, J. L. Uribelarrea, C. Molina-Jouve and J. M. Nicaud, *Yarrowia lipolytica as a model for bio-oil production*, Prog Lipid Res, 2009, **48**, 375-387.

152. A. Beopoulos, Z. Mrozova, F. Thevenieau, M. T. Le Dall, I. Hapala, S. Papanikolaou, T. Chardot and J. M. Nicaud, *Control of lipid accumulation in the yeast Yarrowia lipolytica*, Appl. Environ. Microbiol., 2008, **74**, 7779-7789.

153. A. Anschau, M. C. A. Xavier, S. Hernalsteens and T. T. Franco, *Effect of feeding strategies on lipid production by Lipomyces starkeyi*, Bioresour. Technol., 2014, **157**, 214-222.

154. Z. Gong, Q. Wang, H. Shen, C. Hu, G. Jin and Z. K. Zhao, *Co-fermentation of cellobiose and xylose by Lipomyces starkeyi for lipid production*, Bioresour. Technol., 2012, **117**, 20-24.

155. X. Zhao, X. Kong, Y. Hua, B. Feng and Z. Zhao, *Medium optimization for lipid production through co-fermentation of glucose and xylose by the oleaginous yeast Lipomyces starkeyi*, Eur. J. Lipid Sci. Technol., 2008, **110**, 405-412.

156. X. Yu, J. Zeng, Y. Zheng and S. Chen, *Effect of lignocellulose degradation products on microbial biomass and lipid production by the oleaginous yeast Cryptococcus curvatus*, Process Biochem, 2014, **49**, 457-465.

157. Z. Gong, H. Shen, X. Yang, Q. Wang, H. Xie and Z. K. Zhao, *Lipid production from corn stover by the oleaginous yeast Cryptococcus curvatus*, Biotechnol. Biofuels, 2014, **7**, 158.

158. Y. Cui, J. W. Blackburn and Y. Liang, *Fermentation optimization for the production of lipid by Cryptococcus curvatus: Use of response surface methodology*, Biomass Bioenergy, 2012, **47**, 410-417.

159. G. Christophe, J. L. Deo, V. Kumar, R. Nouaille, P. Fontanille and C. Larroche, *Production of oils from acetic acid by the oleaginous yeast Cryptococcus curvatus*, Appl Biochem Biotechnol, 2012, **167**, 1270-1279.

160. J. Zhang, X. Fang, X.-L. Zhu, Y. Li, H.-P. Xu, B.-F. Zhao, L. Chen and X.-D. Zhang, *Microbial lipid production by the oleaginous yeast Cryptococcus curvatus O3 grown in fed-batch culture*, Biomass Bioenergy, 2011, **35**, 1906-1911.

161. P. A. E. P. Meesters, G. N. M. Huijberts and G. Eggink, *High-cell-density cultivation of the lipid accumulating yeast Cryptococcus curvatus using glycerol as a carbon source*, Appl. Microbiol. Biotechnol., 1996, **45**, 575-579.

162. P. A. E. P. Meesters, H. Van Der Wal, R. Weusthuis and G. Eggink, *Cultivation of the oleaginous yeast Cryptococcus Curvatus in a new reactor with improved mixing and mass transfer characteristics (surer®)*, Biotechnol. Tech., 1996, **10**, 277-282.

163. B. Mast, N. Zöhrens, F. Schmidl, R. Hernandez, W. T. French, N. Merkt, W. Claupein and S. Graeff-Hönninger, *Lipid Production for Microbial Biodiesel by the Oleagenious Yeast Rhodotorula glutinis Using Hydrolysates of Wheat Straw and Miscanthus as Carbon Sources*, Waste Biomass Valorization, 2014, **5**, 955-962.

164. G. Zhang, W. T. French, R. Hernandez, E. Alley and M. Paraschivescu, *Effects of furfural and acetic acid on growth and lipid production from glucose and xylose by Rhodotorula glutinis*, Biomass Bioenergy, 2011, **35**, 734-740.

165. E. R. Easterling, W. T. French, R. Hernandez and M. Licha, *The effect of glycerol as a sole and secondary substrate on the growth and fatty acid composition of Rhodotorula glutinis*, Bioresour. Technol., 2009, **100**, 356-361.

166. F. Xue, J. Miao, X. Zhang, H. Luo and T. Tan, *Studies on lipid production by Rhodotorula glutinis fermentation using monosodium glutamate wastewater as culture medium*, Bioresour. Technol., 2008, **99**, 5923-5927.

167. C. Malisorn and W. Suntornsuk, *Optimization of β-carotene production by Rhodotorula glutinis DM28 in fermented radish brine*, Bioresour. Technol., 2008, **99**, 2281-2287.

168. L. M. Granger, P. Perlot, G. Goma and A. Pareilleux, *Effect of various nutrient limitations on fatty acid production by Rhodotorula glutinis*, Appl. Microbiol. Biotechnol., 1993, **38**, 784-789.

169. L. M. Granger, P. Perlot, G. Goma and A. Pareilleux, *Kinetics of growth and fatty acid production of Rhodotorula glutinis*, Appl. Microbiol. Biotechnol., 1992, **37**, 13-17.

170. X. Yang, G. Jin, Z. Gong, H. Shen, F. Bai and Z. K. Zhao, *Recycling biodiesel-derived glycerol by the oleaginous yeast Rhodosporidium toruloides Y4 through the two-stage lipid production process*, Biochem Eng J, 2014, **91**, 86-91.

171. Q. Huang, Q. Wang, Z. Gong, G. Jin, H. Shen, S. Xiao, H. Xie, S. Ye, J. Wang and Z. K. Zhao, *Effects of selected ionic liquids on lipid production by the oleaginous yeast Rhodosporidium toruloides*, Bioresour. Technol., 2013, **130**, 339-344.

172. J. Ling, S. Nip and H. Shim, *Enhancement of lipid productivity of Rhodosporidium toruloides in distillery wastewater by increasing cell density*, Bioresour. Technol., 2013, **146**, 301-309.

173. H. Shen, Z. Gong, X. Yang, G. Jin, F. Bai and Z. K. Zhao, *Kinetics of continuous cultivation of the oleaginous yeast Rhodosporidium toruloides*, J. Biotechnol., 2013, **168**, 85-89.

174. W. Zhou, Y. Li, Y. Zhang and Z. Zhao, *Energy efficiency evaluation of lipid production by oleaginous yeast Rhodosporidium toruloides*, J. Therm. Anal. Calorim., 2012, **108**, 119-126.

175. X. Zhao, F. Peng, W. Du, C. Liu and D. Liu, *Effects of some inhibitors on the growth and lipid accumulation of oleaginous yeast Rhodosporidium toruloides and preparation of biodiesel by enzymatic transesterification of the lipid*, Bioprocess Biosyst. Eng., 2012, **35**, 993-1004.

176. S. Wu, X. Zhao, H. Shen, Q. Wang and Z. K. Zhao, *Microbial lipid production by Rhodosporidium toruloides under sulfate-limited conditions*, Bioresour. Technol., 2011, **102**, 1803-1807.

177. X. Zhao, S. Wu, C. Hu, Q. Wang, Y. Hua and Z. K. Zhao, *Lipid production from Jerusalem artichoke by Rhodosporidium toruloides Y4*, J. Ind. Microbiol. Biotechnol., 2010, **37**, 581-585.

178. X. Zhao, C. Hu, S. Wu, H. Shen and Z. Zhao, *Lipid production by Rhodosporidium toruloides Y4 using different substrate feeding strategies*, J. Ind. Microbiol. Biotechnol., 2010, **38**, 627-632.

179. C. Hu, X. Zhao, J. Zhao, S. Wu and Z. K. Zhao, *Effects of biomass hydrolysis by-products on oleaginous yeast Rhodosporidium toruloides*, Bioresour. Technol., 2009, **100**, 4843-4847.

180. D. J. Murphy, *The biogenesis and functions of lipid bodies in animals, plants and microorganisms*, Prog Lipid Res, 2001, **40**, 325-438.

181. U. Garscha and E. H. Oliw, *Steric analysis of 8-hydroxy- and 10-hydroxyoctadecadienoic acids and dihydroxyoctadecadienoic acids formed from 8R-hydroperoxyoctadecadienoic acid by hydroperoxide isomerases*, Anal. Biochem., 2007, **367**, 238-246.

182. C. A. Boulton and C. Ratledge, *Correlation of lipid accumulation in yeasts with possession of ATP:citrate lyase*, J. Gen. Microbiol., 1981, **127**, 169-176.

183. F. Lynen and S. Ochoa, *Enzymes of fatty acid metabolism*, Biochim. Biophys. Acta, 1953, **12**, 299-314.

184. J. M. Gancedo, *Yeast carbon catabolite repression*, Microbiol Mol Biol Rev, 1998, **62**, 334-361.

185. L. A. Garay, K. L. Boundy-Mills and J. B. German, *Accumulation of High-Value Lipids in Single-Cell Microorganisms: A Mechanistic Approach and Future Perspectives*, J. Agric. Food Chem., 2014, **62**, 2709-2727.

186. R. Shaw, *The fatty acids of phycomycete fungi, and the significance of the γ-linolenic acid component*, Comp Biochem Physiol, 1966, **18**, 325-331.

187. K. Weinhandl, M. Winkler, A. Glieder and A. Camattari, *Carbon source dependent promoters in yeasts*, Microb Cell Fact, 2014, **13**, 5.

188. C. M. Henderson, W. F. Zeno, L. A. Lerno, M. L. Longo and D. E. Block, *Fermentation Temperature Modulates Phosphatidylethanolamine and Phosphatidylinositol Levels in the Cell Membrane of Saccharomyces cerevisiae*, Appl. Environ. Microbiol., 2013, **79**, 5345-5356.

189. J. Tao, C.-C. Dai, Q.-Y. Yang, X.-Y. Guan and W.-L. Shao, *Production of Biodiesel with Acid Hydrolysate of Populus Euramevicana CV Leaves by Rhodotorula Glutinis*, Int. J. Green Energy, 2010, **7**, 387-396.

190. X. Huang, Y. Wang, W. Liu and J. Bao, *Biological removal of inhibitors leads to the improved lipid production in the lipid fermentation of corn stover hydrolysate by Trichosporon cutaneum*, Bioresour. Technol., 2011, **102**, 9705-9709.

191. A. A. Koutinas, A. Chatzifragkou, N. Kopsahelis, S. Papanikolaou and I. K. Kookos, *Design and techno-economic evaluation of microbial oil production as a renewable resource for biodiesel and oleochemical production*, Fuel, 2014, **116**, 566-577.

192. J. R. Davies, in *Industrial applications of single cell oils*, ed. D. J. K. a. C. Ratledge, American Oil Chemists Society, Boulder, Urbana, IL, Editon edn., 1993, pp. 196-218.

193. J. Blazeck, A. Hill, L. Liu, and H. Alper, *Compositions And Methods For Fungal Lipid Production, US Pat.*, WO/2014/179748, 2014.

194. M. H. Liang and J. G. Jiang, *Advancing oleaginous microorganisms to produce lipid via metabolic engineering technology*, Prog Lipid Res, 2013, **52**, 395-408.

195. J. Davison, *GM plants: Science, politics and EC regulations*, Plant Science, 2010, **178**, 94-98.

196. C. Ratledge and J. P. Wynn, in *Food Biotechnology, Second Edition*, eds. A. Pometto, K. Shetty, G. Paliyath and R. E. Levin, Taylor & Francis, 2nd edn., 2005, pp. 433-472.

197. C. L. Broadhurst and M. Winther, Evening primrose oil: Pharmacological and clinical applications, 2000.

198. Idemitsu, *Idemitsu Agri-Bio Business*, http://www.idemitsu.com/products/agri/about/index.html, Accessed 26/05/2015, 2015.

199. J. T. Bamford, S. Ray, A. Musekiwa, C. van Gool, R. Humphreys and E. Ernst, *Oral evening primrose oil and borage oil for eczema*, The Cochrane database of systematic reviews, 2013, **4**.

200. H. C. Williams, *Evening primrose oil for atopic dermatitis: Time to say goodnight*, BMJ : British Medical Journal, 2003, **327**, 1358-1359.

201. S. P. Hong, P. L. Sharpe, Z. Xue, N. S. Yadav and Q. Zhu, *Primary High eicosapentaenoic acid oils from improved optimized strains of yarrowia lipolytica*, 2010.

202. Z. Xue, P. L. Sharpe, S. P. Hong, N. S. Yadav, D. Xie, D. R. Short, H. G. Damude, R. A. Rupert, J. E. Seip, J. Wang, D. W. Pollak, M. W. Bostick, M. D. Bosak, D. J. Macool, D. H. Hollerbach, H. Zhang, D. M. Arcilla, S. A. Bledsoe, K. Croker, E. F. McCord, B. D. Tyreus, E. N. Jackson and Q. Zhu, *Production of omega-3 eicosapentaenoic acid by metabolic engineering of Yarrowia lipolytica*, Nat. Biotechnol., 2013, **31**, 734-740.

203. F. D. Harzevili and H. Chen, *Microbial Biotechnology: Progress and Trends*, 1st edn., Taylor & Francis, 2014.

204. Verlasso, *Fish in, fish out: conserving wild fish populations*, http://www.verlasso.com/farming/fish-in-fish-out, Accessed 08/07/2015, 2015.

205. L. Kilian, *The economic effects of energy price shocks*, Journal of Economic Literature, 2008, **46**, 871-909.

206. G. Van Calster, W. Vandenberghe and L. Reins, *Research Handbook on Climate Change Mitigation Law*, 1st edn., Edward Elgar Publishing Limited, 2015.

207. Y. Liang, Y. Cui, J. Trushenski and J. W. Blackburn, *Converting crude glycerol derived from yellow grease to lipids through yeast fermentation*, Bioresour. Technol., 2010, **101**, 7581-7586.

208. G. Rødsrud, M. Lersch and A. Sjöde, *History and future of world's most advanced biorefinery in operation*, Biomass Bioenergy, 2012, **46**, 46-59.

209. L. Viikari, J. Vehmaanperä and A. Koivula, *Lignocellulosic ethanol: From science to industry*, Biomass Bioenergy, 2012, **46**, 13-24.

210. J. Larsen, M. Ø. Haven and L. Thirup, *Inbicon makes lignocellulosic ethanol a commercial reality*, Biomass Bioenergy, 2012, **46**, 36-45.

211. E. Gnansounou, *Production and use of lignocellulosic bioethanol in Europe: Current situation and perspectives*, Bioresour. Technol., 2010, **101**, 4842-4850.

212. J. Larsen, M. Petersen, L. Thirup, H. Li and F. Iversen, *The IBUS process - lignocellulosic bioethanol close to a commercial reality*, Chem. Eng. Technol., 2008, **31**, 765 - 772.

213. L. Lynd, M. Laser, D. Bransby, B. Dale, B. Davison, R. Hamilton, M. Himmel, M. Keller, J. McMillan, J. Sheehan and C. Wyman, *How biotech can transform biofuels*, Nat Biotechnol, 2008, **26**, 169 - 172.

214. C. Wyman, *What is (and is not) vital to advancing cellulosic ethanol*, Trends Biotechnol, 2007, **27**, 153 - 157.

215. D. K. Sidiras and E. G. Koukios, *Acid saccharification of ball-milled straw*, Biomass, 1989, **19**, 289-306.

216. M. Galbe and G. Zacchi, *Pretreatment: The key to efficient utilization of lignocellulosic materials*, Biomass Bioenergy, 2012, **46**, 70-78.

217. D. Chiaramonti, M. Prussi, S. Ferrero, L. Oriani, P. Ottonello, P. Torre and F. Cherchi, *Review of pretreatment processes for lignocellulosic ethanol production, and development of an innovative method*, Biomass Bioenergy, 2012, **46**, 25-35.

218. S. McIntosh and T. Vancov, *Optimisation of dilute alkaline pretreatment for enzymatic saccharification of wheat straw*, Biomass Bioenergy, 2011, **35**, 3094-3103.

219. M. Pedersen, K. Johansen and A. Meyer, *Low temperature lignocellulose pretreatment: effects and interactions of pretreatment pH are critical for maximizing enzymatic monosaccharide yields from wheat straw*, Biotechnol. Biofuels, 2011, **4**, 11.

220. J.-W. Lee and T. W. Jeffries, *Efficiencies of acid catalysts in the hydrolysis of lignocellulosic biomass over a range of combined severity factors*, Bioresour. Technol., 2011, **102**, 5884-5890.

221. P. Alvira, E. Tomás-Pejó, M. Ballesteros and M. J. Negro, *Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review*, Bioresour. Technol., 2010, **101**, 4851-4861.

222. N. Mosier, C. Wyman, B. Dale, R. Elander, Y. Y. Lee, M. Holtzapple and M. Ladisch, *Features of promising technologies for pretreatment of lignocellulosic biomass*, Bioresour. Technol., 2005, **96**, 673-686.

223. T. P. Schultz, C. J. Biermann and G. D. McGinnis, *Steam explosion of mixed hardwood chips as a biomass pretreatment*, Ind. Eng. Chem. Prod. Res. Dev., 1983, **22**, 344-348.

224. H. Alizadeh, F. Teymouri, T. I. Gilbert and B. E. Dale, *Pretreatment of switchgrass by ammonia fiber explosion (AFEX)*, Applied Biochemistry and Biotechnology - Part A Enzyme Engineering and Biotechnology, 2005, **124**, 1133-1141.

225. D. G. Macdonald, N. N. Bakhshi, J. F. Mathews, A. Roychowdhury, P. Bajpai and M. Mooyoung, *ALKALI TREATMENT OF CORN STOVER TO IMPROVE SUGAR PRODUCTION BY ENZYMATIC-HYDROLYSIS*, Biotechnol. Bioeng., 1983, **25**, 2067-2076.

226. M. L. Soto, H. Dominguez, M. J. Nunez and J. M. Lema, *ENZYMATIC SACCHARIFICATION OF ALKALI-TREATED SUNFLOWER HULLS*, Bioresour. Technol., 1994, **49**, 53-59.

227. A. Varnai, M. Makela, D. Djajadi, J. Rahikainen, A. Hatakka and L. Viikari, in *Adv Appl Microbiol*, eds. G. M. Gadd and S. Sariaslani, Elsevier Science, Editon edn., 2014, vol. 88.

228. F. M. Medie, G. J. Davies, M. Drancourt and B. Henrissat, *Genome analyses highlight the different biological roles of cellulases*, Nat. Rev. Microbiol., 2012, **10**, 227-U.

229. V. Archambault-Leger, X. Shao and L. R. Lynd, *Integrated analysis of hydrothermal flow through pretreatment*, Biotechnol. Biofuels, 2012, **5**.

230. R. M. Rodríguez-Jasso, S. I. Mussatto, L. Pastrana, C. N. Aguilar and J. A. Teixeira, *Extraction of sulfated polysaccharides by autohydrolysis of brown seaweed Fucus vesiculosus*, J Appl Phycol, 2012, 1-9.

231. Q. Xiang, Y. Lee and R. Torget, *Kinetics of glucose decomposition during dilute-acid hydrolysis of lignocellulosic biomass*, Appl Biochem Biotechnol, 2004, **115**, 1127-1138.

232. Q. Xiang, J. Kim and Y. Y. Lee, *A comprehensive kinetic model for dilute-acid hydrolysis of cellulose*, Appl Biochem Biotechnol, 2003, **106**, 337-352.

233. F. K. Kazi, J. Fortman, R. Anex, G. Kothandaraman, D. Hsu, A. Aden and A. Dutta, *Techno-Economic Analysis of Biochemical Scenarios for Production of Cellulosic Ethanol* National renewable energy authority, Golden, Colorado, US, 2010.

234. Y. Zhao, W. Lu, J. Chen, X. Zhang and H. Wang, *Research progress on hydrothermal dissolution and hydrolysis of lignocellulose and lignocellulosic waste*, Front. Environ. Sci. Eng., 2014, **8**, 151-161.

235. D. D. Macdonald and L. B. Kriksunov, *Probing the chemical and electrochemical properties of SCWO systems*, Electrochim. Acta, 2001, **47**, 775-790.

236. S. Kumar and R. B. Gupta, *Hydrolysis of Microcrystalline Cellulose in Subcritical and Supercritical Water in a Continuous Flow Reactor*, Ind. Eng. Chem. Res., 2008, **47**, 9321-9329.

237. S. G. Allen, L. C. Kam, A. J. Zemann and M. J. Antal, *Fractionation of Sugar Cane with Hot, Compressed, Liquid Water*, Ind. Eng. Chem. Res., 1996, **35**, 2709-2715.

238. S. Brethauer and C. E. Wyman, *Review: Continuous hydrolysis and fermentation for cellulosic ethanol production*, Bioresour. Technol., 2010, **101**, 4862-4874.

239. J. G. Pan and J. S. Rhee, *BIOMASS YIELDS AND ENERGETIC YIELDS OF OLEAGINOUS YEASTS IN BATCH CULTURE*, Biotechnol. Bioeng., 1986, **28**, 112-114.

240. K. Öhgren, R. Bura, G. Lesnicki, J. Saddler and G. Zacchi, *A comparison between simultaneous saccharification and fermentation and separate hydrolysis and fermentation using steam-pretreated corn stover*, Process Biochem, 2007, **42**, 834-839.

241. Z. Kádár, Z. Szengyel and K. Réczey, *Simultaneous saccharification and fermentation (SSF) of industrial wastes for the production of ethanol*, Ind. Crops Prod., 2004, **20**, 103-110.

242. F. Alfani, A. Gallifuoco, A. Saporosi, A. Spera and M. Cantarella, *Comparison of SHF and SSF processes for the bioconversion of steam-exploded wheat straw*, J. Ind. Microbiol. Biotechnol., 2000, **25**, 184-192.

243. T. Hasunuma and A. Kondo, *Development of yeast cell factories for consolidated bioprocessing of lignocellulose to bioethanol through cell surface engineering*, Biotechnol Adv, 2012, **30**, 1207-1218.

244. L. R. Lynd, P. J. Weimer, W. H. van Zyl and I. S. Pretorius, *Microbial Cellulose Utilization: Fundamentals and Biotechnology*, Microbiol Mol Biol Rev, 2002, **66**, 506-577.

245. T. Hasunuma, F. Okazaki, N. Okai, K. Y. Hara, J. Ishii and A. Kondo, *A review of enzymes and microbes for lignocellulosic biorefinery and the possibility of their application to consolidated bioprocessing technology*, Bioresour. Technol., 2013, **135**, 513-522.

246. D. C. la Grange, R. den Haan and W. H. van Zyl, *Engineering cellulolytic ability into bioprocessing organisms*, Appl. Microbiol. Biotechnol., 2010, **87**, 1195-1208.

247. L. R. Lynd, W. H. Van Zyl, J. E. McBride and M. Laser, *Consolidated bioprocessing of cellulosic biomass: An update*, Curr. Opin. Biotechnol., 2005, **16**, 577-583.

248. D. G. Olson, J. E. McBride, A. J. Shaw and L. R. Lynd, *Recent progress in consolidated bioprocessing*, Curr. Opin. Biotechnol., 2012, **23**, 396-405.

249. B. Schuster and M. Chinn, *Consolidated Bioprocessing of Lignocellulosic Feedstocks for Ethanol Fuel Production*, Bioenergy Research, 2013, **6**, 416-435.

250. X. Peng and H. Chen, *Single cell oil production in solid-state fermentation by Microsphaeropsis sp from steam-exploded wheat straw mixed with wheat bran*, Bioresour. Technol., 2008, **99**, 3885-3889.

251. Z. Gong, Q. Wang, H. Shen, L. Wang, H. Xie and Z. K. Zhao, *Conversion of biomass-derived oligosaccharides into lipids*, Biotechnol. Biofuels, 2014, **7**.

252. F. Agbogbo and G. Coward-Kelly, *Cellulosic ethanol production using the naturally occurring xylose-fermenting yeast, Pichia stipitis*, Biotechnol. Lett., 2008, **30**, 1515-1524.

253. Y. H. P. Zhang and L. R. Lynd, *Cellulose utilization by Clostridium thermocellum: Bioenergetics and hydrolysis product assimilation*, Proc. Natl. Acad. Sci. U. S. A., 2005, **102**, 7321-7325.

254. S. Katahira, A. Mizuike, H. Fukuda and A. Kondo, *Ethanol fermentation from lignocellulosic hydrolysate by a recombinant xylose- and cellooligosaccharide-assimilating yeast strain*, Appl. Microbiol. Biotechnol., 2006, **72**, 1136-1143.

255. M. Taccari, L. Canonico, F. Comitini, I. Mannazzu and M. Ciani, *Screening of yeasts for growth on crude glycerol and optimization of biomass production*, Bioresour. Technol., 2012, **110**, 488-495.

256. S. Katahira, Y. Fujita, A. Mizuike, H. Fukuda and A. Kondo, *Construction of a xylan-fermenting yeast strain through codisplay of xylanolytic enzymes on the surface of xylose-utilizing Saccharomyces cerevisiae cells*, Appl. Environ. Microbiol., 2004, **70**, 5407-5414.

257. T. W. Jeffries and Y. S. Jin, *Metabolic engineering for improved fermentation of pentoses by yeasts*, Appl. Microbiol. Biotechnol., 2004, **63**, 495-509.

258. M. Sonderegger, M. Jeppsson, C. Larsson, M. F. Gorwa-Grauslund, E. Boles, L. Olsson, I. Spencer-Martins, B. Hahn-Hagerdal and U. Sauer, *Fermentation*

performance of engineered and evolved xylose-fermenting Saccharomyces cerevisiae strains, Biotechnol. Bioeng., 2004, **87**, 90-98.

259. M. Jeppsson, B. Johansson, B. Hahn-Hagerdal and M. F. Gorwa-Grauslund, *Reduced oxidative pentose phosphate pathway flux in recombinant xylose-utilizing Saccharomyces cerevisiae strains improves the ethanol yield from xylose*, Appl. Environ. Microbiol., 2002, **68**, 1604-1609.

260. B. Hahn-Hagerdal, C. F. Wahlbom, M. Gardonyi, W. H. van Zyl, R. R. Cordero Otero and L. J. Jonsson, *Metabolic engineering of Saccharomyces cerevisiae for xylose utilization*, Advances in biochemical engineering/biotechnology, 2001, **73**, 53-84.

261. M. H. Toivari, A. Aristidou, L. Ruohonen and M. Penttila, *Conversion of xylose to ethanol by recombinant Saccharomyces cerevisiae: Importance of xylulokinase (XKS1) and oxygen availability*, Metab. Eng., 2001, **3**, 236-249.

262. A. Eliasson, C. Christensson, C. F. Wahlbom and B. Hahn-Hagerdal, *Anaerobic xylose fermentation by recombinant Saccharomyces cerevisiae carrying XYL1, XYL2, and XKS1 in mineral medium chemostat cultures*, Appl. Environ. Microbiol., 2000, **66**, 3381-3386.

263. N. W. Y. Ho, Z. D. Chen and A. P. Brainard, *Genetically engineered Sacccharomyces yeast capable of effective cofermentation of glucose and xylose*, Appl. Environ. Microbiol., 1998, **64**, 1852-1859.

264. B. Hahn-Haegerdal, T. Linden, T. Senac and K. Skoog, *Ethanolic fermentation of pentoses in lignocellulose hydrolyzates*, Appl. Biochem. Biotechnol., 1991, **28-29**, 131-144.

265. T. Sakamoto, T. Hasunuma, Y. Hori, R. Yamada and A. Kondo, *Direct ethanol production from hemicellulosic materials of rice straw by use of an engineered yeast strain codisplaying three types of hemicellulolytic enzymes on the surface of xylose-utilizing Saccharomyces cerevisiae cells*, J. Biotechnol., 2012, **158**, 203-210.

266. X. Liang, T. Yoshida and T. Uryu, *Direct saccharification and ethanol fermentation of cello-oligosaccharides with recombinant yeast*, Carbohydr. Polym., 2013, **91**, 157-161.

267. T. Fujii, G. Yu, A. Matsushika, A. Kurita, S. Yano, K. Murakami and S. Sawayama, *Ethanol production from xylo-oligosaccharides by xylose-fermenting Saccharomyces cerevisiae expressing β-xylosidase*, Bioscience, Biotechnology and Biochemistry, 2011, **75**, 1140-1146.

268. K. Okano, Q. Zhang, S. Yoshida, T. Tanaka, C. Ogino, H. Fukuda and A. Kondo, *D-lactic acid production from cellooligosaccharides and β-glucan using l-LDH gene-deficient and endoglucanase-secreting Lactobacillus plantarum*, Appl. Microbiol. Biotechnol., 2010, **85**, 643-650.

269. T. Murai, M. Ueda, T. Kawaguchi, M. Arai and A. Tanaka, *Assimilation of cellooligosaccharides by a cell surface-engineered yeast expressing β-glucosidase and carboxymethylcellulase from Aspergillus aculeatus*, Appl. Environ. Microbiol., 1998, **64**, 4857-4861.

270. L. J. Jönsson, B. Alriksson and N. O. Nilvebrant, *Bioconversion of lignocellulose: Inhibitors and detoxification*, Biotechnol. Biofuels, 2013, **6**.

271. W. Parawira and M. Tekere, *Biotechnological strategies to overcome inhibitors in lignocellulose hydrolysates for ethanol production: review*, Crit Rev Biotechnol, 2011, **31**, 20-31.

272. E. Palmqvist and B. Hahn-Hägerdal, *Fermentation of lignocellulosic hydrolysates. II: Inhibitors and mechanisms of inhibition*, Bioresour. Technol., 2000, **74**, 25-33.

273. E. C. van der Pol, R. R. Bakker, P. Baets and G. Eggink, *By-products resulting from lignocellulose pretreatment and their inhibitory effect on fermentations for (bio)chemicals and fuels*, Appl. Microbiol. Biotechnol., 2014, **98**, 9579-9593.

274. C. Luo, D. L. Brink and H. W. Blanch, *Identification of potential fermentation inhibitors in conversion of hybrid poplar hydrolyzate to ethanol*, Biomass Bioenergy, 2002, **22**, 125-138.

275. S. Larsson, E. Palmqvist, B. Hahn-Hägerdal, C. Tengborg, K. Stenberg, G. Zacchi and N. O. Nilvebrant, *The generation of fermentation inhibitors during dilute acid hydrolysis of softwood*, Enzyme Microb. Technol., 1999, **24**, 151-159.

276. E. Palmqvist, H. Grage, N. Q. Meinander and B. Hahn-Hägerdal, *Main and interaction effects of acetic acid, furfural, and p-hydroxybenzoic acid on growth and ethanol productivity of yeasts*, Biotechnol. Bioeng., 1999, **63**, 46-55.

277. I. C. Roberto, L. S. Lacis, M. F. S. Barbosa and I. M. de Mancilha, *Utilization of sugar cane bagasse hemicellulosic hydrolysate by pichia stipitis for the production of ethanol*, Process Biochem, 1991, **26**, 15-21.

278. X. Chen, Z. Li, X. Zhang, F. Hu, D. D. Y. Ryu and J. Bao, *Screening of oleaginous yeast strains tolerant to lignocellulose degradation compounds*, Appl Biochem Biotechnol, 2009, **159**, 591-604.

279. M. J. Taherzadeh, L. Gustafsson, C. Niklasson and G. Liden, *Conversion of furfural in aerobic and anaerobic batch fermentation of glucose by Saccharomyces cerevisiae*, J. Biosci. Bioeng., 1999, **87**, 169-174.

280. M.-Z. Ding, X. Wang, W. Liu, J.-S. Cheng, Y. Yang and Y.-J. Yuan, *Proteomic Research Reveals the Stress Response and Detoxification of Yeast to Combined Inhibitors*, PLoS One, 2012, **7**, e43474.

281. E. Lohmeier-Vogel, C. Sopher and H. Lee, *Intracellular acidification as a mechanism for the inhibition by acid hydrolysis-derived inhibitors of xylose fermentation by yeasts*, J Ind Microbiol Biotechnol, 1998, **20**, 75 - 81.

282. C. Huang, M.-h. Zong, H. Wu and Q.-p. Liu, *Microbial oil production from rice straw hydrolysate by Trichosporon fermentans*, Bioresour. Technol., 2009, **100**, 4535-4538.

283. J. Chen, Y. Zhang, Y. Wang, X. Ji, L. Zhang, X. Mi and H. Huang, *Removal of inhibitors from lignocellulosic hydrolyzates by vacuum membrane distillation*, Bioresour. Technol., 2013, **144**, 680-683.

284. A. Martinez, M. E. Rodriguez, S. W. York, J. F. Preston and L. O. Ingram, *Effects of Ca(OH)(2) treatments ("overliming") on the composition and toxicity of bagasse hemicellulose hydrolysates*, Biotechnol. Bioeng., 2000, **69**, 526-536.

285. G. D. Ulrich, *A guide to chemical engineering process design and economics*, 1st edn., Wiley, New York 1984.

286. Z. Lewis Liu, M. Ma and M. Song, *Evolutionarily engineered ethanologenic yeast detoxifies lignocellulosic biomass conversion inhibitors by reprogrammed pathways*, Mol. Genet. Genomics, 2009, **282**, 233-244.

287. Z. L. Liu, P. J. Slininger, B. S. Dien, M. A. Berhow, C. P. Kurtzman and S. W. Gorsich, *Adaptive response of yeasts to furfural and 5-hydroxymethylfurfural and new chemical evidence for HMF conversion to 2,5-bis-hydroxymethlfuran*, J. Ind. Microbiol. Biotechnol., 2004, **31**, 345-352.

288. Z. L. Liu, *Molecular mechanisms of yeast tolerance and in situ detoxification of lignocellulose hydrolysates*, Appl. Microbiol. Biotechnol., 2011, **90**, 809-825.

289. S. Larsson, A. Quintana-Sainz, A. Reimann, N. O. Nilvebrant and L. J. Jonsson, *Influence of lignocellulose-derived aromatic compounds on oxygen-limited growth and ethanolic fermentation by Saccharomyces cerevisiae*, Appl Biochem Biotechnol, 2000, **84-6**, 617-632.

290. C. Huang, H. Wu, Z. J. Liu, J. Cai, W. Y. Lou and M. H. Zong, *Effect of organic acids on the growth and lipid accumulation of oleaginous yeast Trichosporon fermentans*, Biotechnol. Biofuels, 2012, **5**.

291. C. Huang, H. Wu, L. p. Liu, W. y. Lou and M. h. Zong, *Effects of Alcohol Compounds on the Growth and Lipid Accumulation of Oleaginous Yeast Trichosporon fermentans*, PLoS One, 2012, **7**.

292. C. Huang, H. Wu, Q.-p. Liu, Y.-y. Li and M.-h. Zong, *Effects of Aldehydes on the Growth and Lipid Accumulation of Oleaginous Yeast Trichosporon fermentans*, J. Agric. Food Chem., 2011, **59**, 4606-4613.

293. D. J. Schell, C. J. Riley, N. Dowe, J. Farmer, K. N. Ibsen, M. F. Ruth, S. T. Toon and R. E. Lumpkin, *A bioethanol process development unit: Initial operating experiences and results with a corn fiber feedstock*, Bioresour. Technol., 2004, **91**, 179-188.

294. E. Albers, E. Johansson, C. J. Franzen and C. Larsson, *Selective suppression of bacterial contaminants by process conditions during lignocellulose based yeast fermentations*, Biotechnol. Biofuels, 2011, **4**.

295. D. J. Schell, N. Dowe, K. N. Ibsen, C. J. Riley, M. F. Ruth and R. E. Lumpkin, *Contaminant occurrence, identification and control in a pilot-scale corn fiber to ethanol conversion process*, Bioresour. Technol., 2007, **98**, 2942-2948.

296. M. Beckner, M. L. Ivey and T. G. Phister, *Microbial contamination of fuel ethanol fermentations*, Lett Appl Microbiol, 2011, **53**, 387-394.

297. Y. C. Sharma, B. Singh and J. Korstad, *A critical review on recent methods used for economically viable and eco-friendly development of microalgae as a potential feedstock for synthesis of biodiesel*, Green Chem., 2011, **13**, 2993-3006.

298. Z. Chi, Y. Zheng, A. Jiang and S. Chen, *Lipid production by culturing oleaginous yeast and algae with food waste and municipal wastewater in an integrated process*, Appl Biochem Biotechnol, 2011, **165**, 442-453.

299. K. Bischoff, K. Skinner-Nemec and T. Leathers, *Antimicrobial susceptibility of Lactobacillus species isolated from commercial ethanol plants*, J. Ind. Microbiol. Biotechnol., 2007, **34**, 739-744.

300. P. A. Vandenbergh, *Lactic acid bacteria, their metabolic products and interference with microbial growth*, FEMS Microbiol. Rev., 1993, **12**, 221-237.

301. C. Laluce, A. C. G. Schenberg, J. C. M. Gallardo, L. F. C. Coradello and S. R. Pombeiro-Sponchiado, *Advances and Developments in Strategies to Improve Strains of Saccharomyces cerevisiae and Processes to Obtain the Lignocellulosic Ethanol–A Review*, Appl Biochem Biotechnol, 2012, **166**, 1908-1926.

302. P. Turner, G. Mamo and E. N. Karlsson, *Potential and utilization of thermophiles and thermostable enzymes in biorefining*, Microb Cell Fact, 2007, **6**.

303. S. Kodama, H. Nakanishi, T. A. T. P. Thalagala, N. Isono and M. Hisamatsu, *A wild and tolerant yeast suitable for ethanol fermentation from lignocellulose*, J. Biosci. Bioeng., 2013, **115**, 557-561.

304. S. S. Dasilva and A. S. Afschar, *Microbial-Production Of Xylitol From D-Xylose Using Candida-Tropicalis*, Bioprocess. Eng., 1994, **11**, 129-134.

305. Z. Kadar, S. F. Maltha, Z. Szengyel, K. Reczey and W. De Laat, *Ethanol fermentation of various pretreated and hydrolyzed substrates at low initial pH*, Appl Biochem Biotechnol, 2007, **137**, 847-858.

306. C. N. Economou, G. Aggelis, S. Pavlou and D. V. Vayenas, *Single cell oil production from rice hulls hydrolysate*, Bioresour. Technol., 2011, **102**, 9737-9742.

307. Z. Ruan, M. Zanotti, Y. Zhong, W. Liao, C. Ducey and Y. Liu, *Co-hydrolysis of lignocellulosic biomass for microbial lipid accumulation*, Biotechnol. Bioeng., 2013, **110**, 1039-1049.

308. J. Lian, M. Garcia-Perez, R. Coates, H. Wu and S. Chen, *Yeast fermentation of carboxylic acids obtained from pyrolytic aqueous phases for lipid production*, Bioresour. Technol., 2012, **118**, 177-186.

309. M. Enshaeieh, A. Abdoli, M. Madani and M. Bayat, *Recycling of lignocellulosic waste materials to produce high-value products: single cell oil and xylitol*, Int. J. Environ. Sci. Technol., 2015, **12**, 837-846.

310. W. Liu, Y. Wang, Z. Yu and J. Bao, *Simultaneous saccharification and microbial lipid fermentation of corn stover by oleaginous yeast Trichosporon cutaneum*, Bioresour. Technol., 2012, **118**, 13-18.

311. X. Chen, C. Huang, L. Xiong, X. Chen and L. Ma, *Microbial oil production from Corncob acid hydrolysate by Trichosporon cutaneum*, Biotechnol. Lett., 2012, **34**, 1025-1028.

312. X. Yu, Y. Zheng, X. Xiong and S. Chen, *Co-utilization of glucose, xylose and cellobiose by the oleaginous yeast Cryptococcus curvatus*, Biomass Bioenergy, 2014, **71**, 340-349.

313. Z. Gong, H. Shen, Q. Wang, X. Yang, H. Xie and Z. K. Zhao, *Efficient conversion of biomass into lipids by using the simultaneous saccharification and enhanced lipid production process*, Biotechnol. Biofuels, 2013, **6**.

314. A. J. Kluyver, J. P. Vanderwalt and A. J. Vantriet, *Pulcherrimin, The Pigment Of Candida Pulcherrima*, Proc. Natl. Acad. Sci. U. S. A., 1953, **39**, 583-593.

315. C. o. life, *Primary Catalogue of Life: 2015 Annual Checklist*, ed. C. o. Life, Species 2000 Secretariat, Leiden, The Netherlands, 2015, vol. 2015.

316. G. A. Farris, I. Mannazzu and M. Budroni, *Identification Of Killer Factor In The Yeast Genus Metschnikowia*, Biotechnol. Lett., 1991, **13**, 297-298.

317. C. Roberts, *The Effect Of Iron And Other Factors On The Production Of Pigment By The Yeast Torulopsis Pulcherrima*, Am J Bot, 1946, **33**, 237-244.

318. D. Saravanakumar, A. Ciavorella, D. Spadaro, A. Garibaldi and M. L. Gullino, *Metschnikowia pulcherrima strain MACH1 outcompetes Botrytis cinerea, Alternaria alternata and Penicillium expansum in apples through iron depletion*, Postharvest Biol. Technol., 2008, **49**, 121-128.

319. O. Csutak, T. Vassu, I. Sarbu, I. Stoica and P. Cornea, *Antagonistic activity of three newly isolated yeast strains from the surface of fruits*, Food Technol Biotechnol, 2013, **51**, 70-77.

320. V. Savini, M. Hendrickx, M. Sisti, G. Masciarelli, M. Favaro, C. Fontana, L. Pitzurra, D. Arzeni, D. Astolfi, C. Catavitello, E. Polilli, C. Farina, P. Fazii, D. D'Antonio and D. Stubbe, *An atypical, pigment-producing Metschnikowia strain from a leukaemia patient*, Med Mycol, 2013, **51**, 438-443.

321. L. Bereczki, N. Bartha, S. Kocsubé, J. Sóki, G. Lengyel, G. Tálosi, K. Máder, J. Deák and I. Dóczi, *Fungaemia caused by Candida pulcherrima*, Med Mycol, 2012, **50**, 522-524.

322. W. P. Kennedy, L. J. Milne, W. Blyth and G. K. Crompton, *Two unusual organisms, Aspergillus terreus and Metschnikowia pulcherrima, associated with the lung disease of ankylosing spondylitis*, Thorax, 1972, **27**, 604-610.

323. F. Santamauro, F. M. Whiffin, R. J. Scott and C. J. Chuck, *Low-cost lipid production by an oleaginous yeast cultured in non-sterile conditions using model waste resources*, Biotechnol. Biofuels, 2014, **7**.

324. E. G. Bligh and W. J. Dyer, *A rapid method of total lipid extraction and purification*, Can J Biochem Physiol, 1959, **37**, 911-917.

325. B. Freedman, R. O. Butterfield and E. H. Pryde, *Transesterification kinetics of soybean oil 1*, J. Am. Oil Chem. Soc., 1986, **63**, 1375-1380.

326. D. Brindha, S. Vinodhini and K. Alarmelumangai, *Extraction and characterization of fiber from three plant species of the genus Cleome, L*, Asian J. Exp. Biol. Sci., 2013, **4**, 69-73.

327. J. A. Perez, A. Gonzalez, J. M. Oliva, I. Ballesteros and P. Manzanares, *Effect of process variables on liquid hot water pretreatment of wheat straw for bioconversion to fuel-ethanol in a batch reactor*, J. Chem. Technol. Biotechnol., 2007, **82**, 929-938.

328. F. Mariotti, D. Tome and P. P. Mirand, *Converting nitrogen into Protein - Beyond 6.25 and Jones' factors*, Crit Rev Food Sci Nutr, 2008, **48**, 177-184.

329. C. V. González López, M. d. C. C. García, F. G. A. Fernández, C. S. Bustos, Y. Chisti and J. M. F. Sevilla, *Protein measurements of microalgal and cyanobacterial biomass*, Bioresour. Technol., 2010, **101**, 7587-7591.

330. D. Spadaro, D. Zhang, A. Garibaldi and M. L. Gullino, *Primary The role of competition for iron and cell wall degrading enzymes in mechanism of action of postharvest biocontrol agents*, 2011, vol. 905, pp. 87-102.

331. H. V. Nguyen and G. Panon, *The yeast Metschnikowia pulcherrima has an inhibitory effect against various yeast species*, Sci Aliments, 1998, **18**, 515-526.

332. J. Larsen, M. O. Petersen, L. Thirup, H. W. Li and F. K. Iversen, *The IBUS process - Lignocellulosic bioethanol close to a commercial reality*, Chem. Eng. Technol., 2008, **31**, 765-772.

333. Y. Xu, R. H. Wang, A. A. Koutinas and C. Webb, *Microbial biodegradable plastic production from a wheat-based biorefining strategy*, Process Biochem. (Amsterdam, Neth.), 2010, **45**, 153-163.

334. C. Weber, A. Farwick, F. Benisch, D. Brat, H. Dietz, T. Subtil and E. Boles, *Trends and challenges in the microbial production of lignocellulosic bioalcohol fuels*, Appl. Microbiol. Biotechnol., 2010, **87**, 1303-1315.

335. P. K. Bajwa, T. Shireen, F. D'Aoust, D. Pinel, V. J. J. Martin, J. T. Trevors and H. Lee, *Mutants of the Pentose-Fermenting Yeast Pichia stipitis With Improved Tolerance to Inhibitors in Hardwood Spent Sulfite Liquor*, Biotechnol. Bioeng., 2009, **104**, 892-900.

336. M. Fernandez, J. F. Ubeda and A. I. Briones, *Typing of non-Saccharomyces yeasts with enzymatic activities of interest in wine-making*, Int J Food Microbiol, 2000, **59**, 29-36.

337. P. González-Pombo, G. Pérez, F. Carrau, J. Guisán, F. Batista-Viera and B. Brena, *One-step purification and characterization of an intracellular β-glucosidase from Metschnikowia pulcherrima*, Biotechnol. Lett., 2008, **30**, 1469-1475.

338. P. Manzanares, D. Ramon and A. Querol, *Screening of non-Saccharomyces wine yeasts for the production of beta-D-xylosidase activity*, Int J Food Microbiol, 1999, **46**, 105-112.

339. G. H. Fleet, *Yeasts in Food and Beverages*, 1st edn., Springer, Berlin, 2006.

340. T. Cadena, F. Prada, A. Perea and H. M. Romero, *Lipase activity, mesocarp oil content, and iodine value in oil palm fruits of Elaeis guineensis, Elaeis oleifera, and the interspecific hybrid O×G (E. oleifera × E. guineensis)*, J. Sci. Food Agric., 2013, **93**, 674-680.

341. A. T. W. M. Hendriks and G. Zeeman, *Pretreatments to enhance the digestibility of lignocellulosic biomass*, Bioresour. Technol., 2009, **100**, 10-18.

342. Y. Sun and J. Cheng, *Hydrolysis of lignocellulosic materials for ethanol production: A review*, Bioresour. Technol., 2002, **83**, 1-11.

343. A. Banerji, M. Balakrishnan and V. V. N. Kishore, *Low severity dilute-acid hydrolysis of sweet sorghum bagasse*, Appl. Energy, 2013, **104**, 197-206.

344. A. C. De Souza, T. Rietkerk, C. G. M. Selin and P. P. Lankhorst, *A robust and universal NMR method for the compositional analysis of polysaccharides*, Carbohydr. Polym., 2013, **95**, 657-663.

345. W. Mangunwardoyo, Aprilismulan, A. Oetari and W. Sjamsuridzal, *Screening cellulose activity of yeast isolated from soil, sediment and water river from Taman Nasional Gunung Halimun, West Java, Indonesia*, Malaysian Journal of Microbiology, 2011, **7**, 210-216.

346. M. J. Lopez, M. d. C. Vargas-García, F. Suárez-Estrella, N. N. Nichols, B. S. Dien and J. Moreno, *Lignocellulose-degrading enzymes produced by the ascomycete Coniochaeta ligniaria and related species: Application for a lignocellulosic substrate treatment*, Enzyme Microb. Technol., 2007, **40**, 794-800.

347. A. Amore and V. Faraco, *Potential of fungi as category I Consolidated BioProcessing organisms for cellulosic ethanol production*, Renewable Sustainable Energy Rev., 2012, **16**, 3286-3301.

348. E. Tomas-Pejo, P. Alvira, M. Ballesteros and M. J. Negro, *Pretreatment Technologies for Lignocellulose-to-Bioethanol Conversion*, Biofuels: Alternative Feedstocks and Conversion Processes, 2011, 149-176.

349. J. A. Perez, I. Ballesteros, M. Ballesteros, F. Saez, M. J. Negro and P. Manzanares, *Optimizing Liquid Hot Water pretreatment conditions to enhance sugar recovery from wheat straw for fuel-ethanol production*, Fuel, 2008, **87**, 3640-3647.

350. L. Perez-Cantu, A. Schreiber, F. Schütt, B. Saake, C. Kirsch and I. Smirnova, *Comparison of pretreatment methods for rye straw in the second generation biorefinery: Effect on cellulose, hemicellulose and lignin recovery*, Bioresour. Technol., 2013, **142**, 428-435.

351. C. Zetzl, K. Gairola, C. Kirsch, L. Perez-Cantu and I. Smirnova, *High Pressure Processes in Biorefineries*, Chem. Ing. Tech., 2011, **83**, 1016-1025.

352. S. Wu, C. Hu, G. Jin, X. Zhao and Z. K. Zhao, *Phosphate-limitation mediated lipid production by Rhodosporidium toruloides*, Bioresour. Technol., 2010, **101**, 6124-6129.

353. S. Bandhu, D. Dasgupta, J. Akhter, P. Kanaujia, S. K. Suman, D. Agrawal, S. Kaul, D. K. Adhikari and D. Ghosh, *Statistical design and optimization of single cell oil production from sugarcane bagasse hydrolysate by an oleaginous yeast Rhodotorula sp IIP-33 using response surface methodology*, Springerplus, 2014, **3**.

354. Y. Cui, J. W. Blackburn and Y. Liang, title, 10AIChE - 2010 AIChE Annual Meeting, Conference Proceedings, 2010.

355. C. J. Chuck, D. Lou-Hing, R. Dean, L. A. Sargeant, R. J. Scott and R. W. Jenkins, *Simultaneous microwave extraction and synthesis of fatty acid methyl ester from the oleaginous yeast Rhodotorula glutinis*, Energy, 2014, **69**, 446-454.

356. A. K. Kaboré, E. Olmos, F. Blanchard, M. Fick, S. Delaunay and E. Guedon, *Impact of gas–liquid mass transfer on organic acids production by Corynebacterium glutamicum in unbaffled shake flasks*, Biochem Eng J, 2015, **101**, 237-247.

357. D. Spadaro, A. Ciavorella, Z. Dianpeng, A. Garibaldi and M. L. Gullino, *Effect of culture media and pH on the biomass production and biocontrol efficacy of a Metschnikowia pulcherrima strain to be used as a biofungicide for postharvest disease control*, Can. J. Microbiol., 2010, **56**, 128-137.

358. G. Najafpour, *Biochemical Engineering and Biotechnology*, 1st edn., Elsevier Science, 2015.

359. J. Fan, M. De Bruyn, Z. Zhu, V. Budarin, M. Gronnow, L. D. Gomez, D. Macquarrie and J. Clark, *Microwave-enhanced formation of glucose from cellulosic waste*, Chem. Eng. Process., 2013, **71**, 37-42.

360. J. Fan, M. De Bruyn, V. L. Budarin, M. J. Gronnow, P. S. Shuttleworth, S. Breeden, D. J. Macquarrie and J. H. Clark, *Direct Microwave-Assisted Hydrothermal Depolymerization of Cellulose*, J. Am. Chem. Soc., 2013, **135**, 11728-11731.

361. C. O. Kappe, *Controlled microwave heating in modern organic synthesis*, Angewandte Chemie-International Edition, 2004, **43**, 6250-6284.

362. D. E. Clark and W. H. Sutton, *Microwave processing of materials*, Annu. Rev. Mater. Sci., 1996, **26**, 299-331.

363. A. Fan, *Primary*, ed. F. M. Whiffin, 2014.

364. R. C. Sun, J. M. Lawther and W. B. Banks, *Fractional and structural characterization of wheat straw hemicelluloses*, Carbohydr. Polym., 1996, **29**, 325-331.

365. J. M. Clemente-Jimenez, L. Mingorance-Cazorla, S. Martinez-Rodriguez, F. J. L. Heras-Vazquez and F. Rodriguez-Vico, *Molecular characterization and oenological properties of wine yeasts isolated during spontaneous fermentation of six varieties of grape must*, Food Microbiol., 2004, **21**, 149-155.

366. P. Domizio, C. Romani, L. Lencioni, F. Comitini, M. Gobbi, I. Mannazzu and M. Ciani, *Outlining a future for non-Saccharomyces yeasts: Selection of putative spoilage wine strains to be used in association with Saccharomyces cerevisiae for grape juice fermentation*, Int J Food Microbiol, 2011, **147**, 170-180.

367. M. Kordowska-Wiater, *Production of arabitol by yeasts: current status and future prospects*, J Appl Microbiol, 2015, **119**, 303-314.

368. M. M. W. Etschmann, W. Bluemke, D. Sell and J. Schrader, *Biotechnological production of 2-phenylethanol*, Appl. Microbiol. Biotechnol., 2002, **59**, 1-8.

369. J. Warr, S. Fraser and O. Grouault, *Primary Sensitive skin perfumes*, 2008.

370. H. R. G and L. J. E, *Primary Method of making aralkyl iodides and corresponding alcohols*, 1943.

371. I. Mazarrasa, Y. S. Olsen, E. Mayol, N. Marbà and C. M. Duarte, *Global unbalance in seaweed production, research effort and biotechnology markets*, Biotechnol Adv, 2014, **32**, 1028-1036.

372. T. Bastian, D. Stokes, J. E. Kelleher, G. C. Hays, J. Davenport and T. K. Doyle, *Fisheries bycatch data provide insights into the distribution of the mauve stinger (Pelagia noctiluca) around Ireland*, ICES Journal of Marine Science: Journal du Conseil, 2011, **68**, 436-443.

373. P. Glampedaki and V. Dutschk, *Stability studies of cosmetic emulsions prepared from natural products such as wine, grape seed oil and mastic resin*, "Colloids Surf., A ", 2014, **460**, 306-311.

374. Y. Cui and Y. Liang, *Direct transesterification of wet Cryptococcus curvatus cells to biodiesel through use of microwave irradiation*, Appl. Energy, 2014, **119**, 438-444.

375. S. Worrasinchai, M. Suphantharika, S. Pinjai and P. Jamnong, *beta-glucan prepared from spent brewer's yeast as a fat replacer in mayonnaise*, Food Hydrocolloids, 2006, **20**, 68-78.

376. Z. Burkus and F. Temelli, *Stabilization of emulsions and foams using barley beta-glucan*, Food Res Int, 2000, **33**, 27-33.

377. S. Thammakiti, M. Suphantharika, T. Phaesuwan and C. Verduyn, *Preparation of spent brewer's yeast β-glucans for potential applications in the food industry*, Int. J. Food Sci. Technol., 2004, **39**, 21-29.

378. D. Bais, A. Trevisan, R. Lapasin, P. Partal and C. Gallegos, *Rheological characterization of polysaccharide–surfactant matrices for cosmetic O/W emulsions*, J. Colloid Interface Sci., 2005, **290**, 546-556.

379. I. B. Ivanov, E. S. Basheva, T. D. Gurkov, A. D. Hadjiiski, L. N. Arnaudov, N. D. Vassileva, S. S. Tcholakova and B. E. Campbell, in *Food Colloids: Fundamentals of Formulation*, eds. E. Dickinson and R. Miller, Editon edn., 2001, pp. 73-90.

380. M. Hu, D. J. McClements and E. A. Decker, *Impact of chelators on the oxidative stability of whey protein isolate-stabilized oil-in-water emulsions containing omega-3 fatty acids*, Food Chem., 2004, **88**, 57-62.

381. C. Amine, J. Dreher, T. Helgason and T. Tadros, *Investigation of emulsifying properties and emulsion stability of plant and milk proteins using interfacial tension and interfacial elasticity*, Food Hydrocolloids, 2014, **39**, 180-186.

382. B. Esteban, J.-R. Riba, G. Baquero, R. Puig and A. Rius, *Characterization of the surface tension of vegetable oils to be used as fuel in diesel engines*, Fuel, 2012, **102**, 231-238.

383. Á. Ábraham, R. A. Campbell and I. Varga, *New Method to Predict the Surface Tension of Complex Synthetic and Biological Polyelectrolyte/Surfactant Mixtures*, Langmuir, 2013, **29**, 11554-11559.

384. H. Fauser, R. von Klitzing and R. A. Campbell, *Surface Adsorption of Oppositely Charged C14TAB-PAMPS Mixtures at the Air/Water Interface and the Impact on Foam Film Stability*, The Journal of Physical Chemistry B, 2015, **119**, 348-358.

385. G. Savary, M. Grisel and C. Picard, *Impact of emollients on the spreading properties of cosmetic products: A combined sensory and instrumental characterization*, "Colloids Surf., B ", 2013, **102**, 371-378.

386. P. D. Darbre and P. W. Harvey, *Paraben esters: review of recent studies of endocrine toxicity, absorption, esterase and human exposure, and discussion of potential human health risks*, J. Appl. Toxicol., 2008, **28**, 561-578.

387. S. Luebberding, N. Krueger and M. Kerscher, *Skin physiology in men and women: in vivo evaluation of 300 people including TEWL, SC hydration, sebum content and skin surface pH*, Int J Cosmet Sci, 2013, **35**, 477-483.

388. D. Swatschek, W. Schatton, J. Kellermann, W. E. G. MullerMüller and J. Kreuter, *Marine sponge collagen: isolation, characterization and effects on the skin parameters surface-pH, moisture and sebum*, Eur J Pharm Biopharm, 2002, **53**, 107-113.

389. C. D. Ampatzidis, E. M. A. Varka and T. D. Karapantsios, *Interfacial activity of amino acid-based glycerol ether surfactants and their performance in stabilizing O/W cosmetic emulsions*, "Colloids Surf., A ", 2014, **460**, 176-183.

390. S. Tcholakova, N. D. Denkov, I. B. Ivanov and B. Campbell, *Coalescence stability of emulsions containing globular milk proteins*, Adv. Colloid Interface Sci., 2006, **123**, 259-293.

391. S. Tcholakova, N. D. Denkov, D. Sidzhakova, I. B. Ivanov and B. Campbell, *Interrelation between drop size and protein adsorption at various emulsification conditions*, Langmuir, 2003, **19**, 5640-5649.

392. M. Bellasio, D. Mattanovich, M. Sauer and H. Marx, *Organic acids from lignocellulose: Candida lignohabitans as a new microbial cell factory*, J. Ind. Microbiol. Biotechnol., 2015, **42**, 681-691.

393. N. Y. Chen, E. F. Kondis and S. Srinivasan, *Low-Pressure Airlift Fermenter For Single Cell Protein-Production .1. Design And Oxygen-Transfer Studies*, Biotechnol. Bioeng., 1987, **29**, 414-420.

394. H.-P. Luo and M. H. Al-Dahhan, *Macro-mixing in a draft-tube airlift bioreactor*, Chem. Eng. Sci., 2008, **63**, 1572-1585.

10 Appendices

10.1 Appendix 1

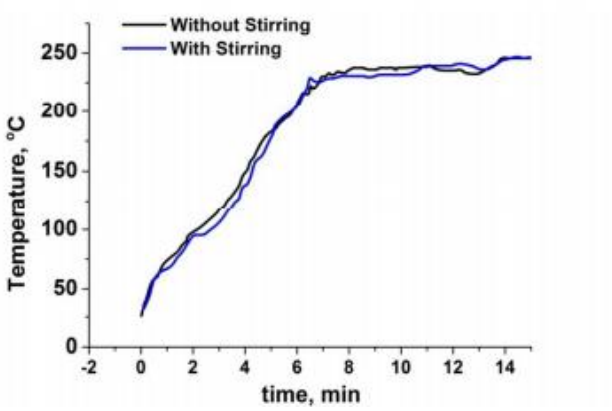


Figure 10.1. Example temperature profile with time of CEM MARS 6 microwave reactor on microcrystalline cellulose model with (blue line) and without (black line) stirring. Reprinted with permission from Fan *et al.*[360] Copyright 2013 American Chemical Society.

10.2 Appendix 2

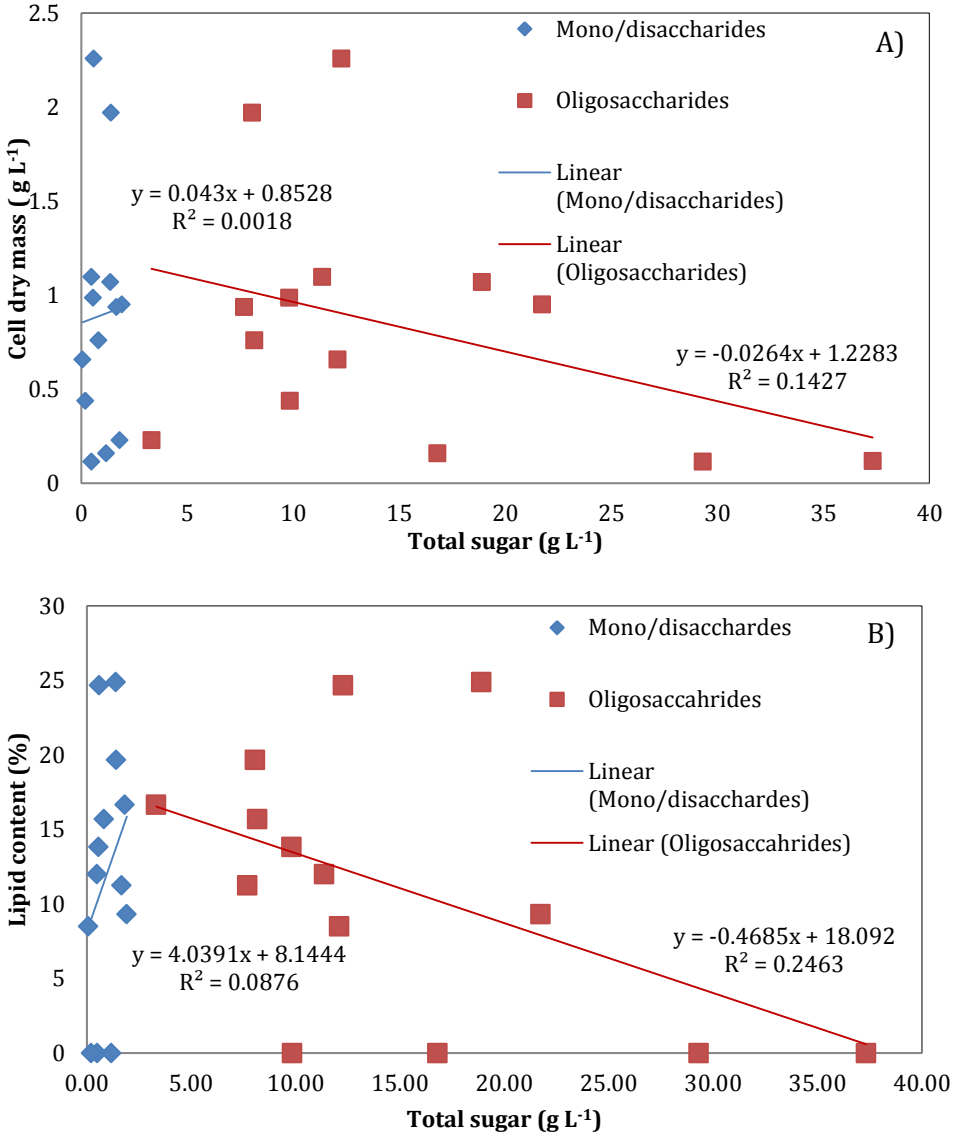


Figure 10.2. Cell dry mass vs sugar concentration (inhibitors under 1 g L^{-1}) showing no correlation between mono or oligosaccharide concentrations and A) cell dry mass and B) lipid content for *M. pulcherrima* grown on microwave hydrolysates.

10.3 Appendix 3

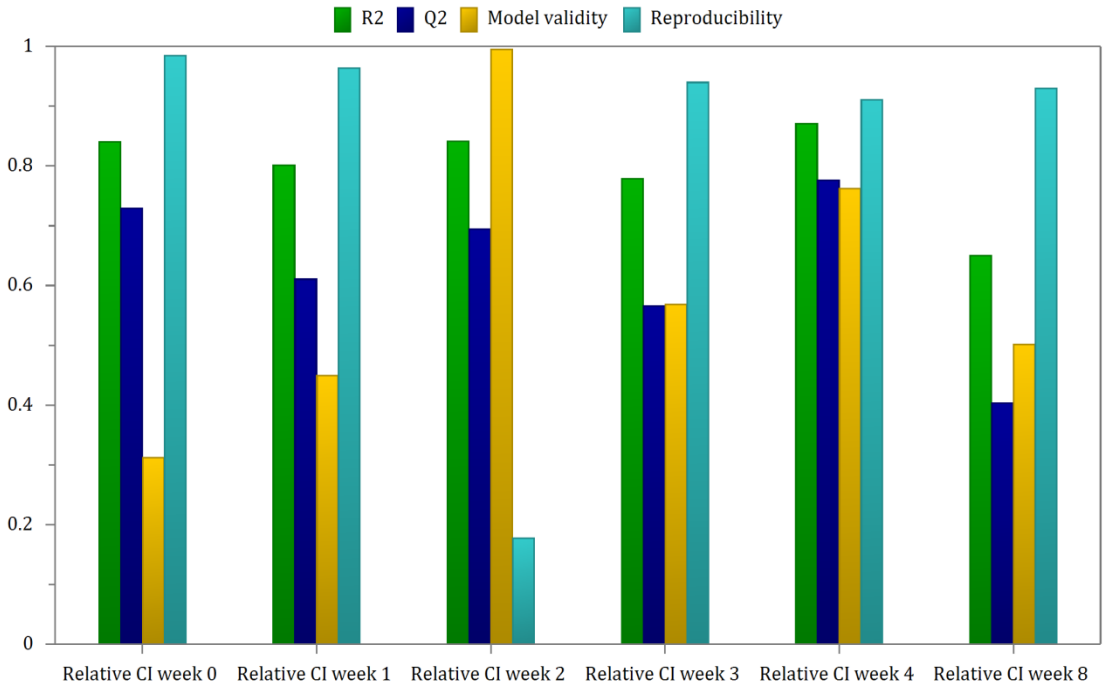


Figure 10.3. Measures of model validity for relative creaming index at each time point.